Hort Innovation

Final Report

Pathogens and other factors contributing to dark staining on pistachio shells

Project leader:

Richard Porter and Dr Belinda Rawnsley

Delivery partner:

AgXtra

Project code:

PS16002

Project:

Pathogens and other factors contributing to dark staining on pistachio shells (PS16002)

Disclaimer:

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Content

Summary

In Australia, the pistachio (*Pistachia vera* L.) industry is projected to be worth $25 million by 2020, with a current planted area of approximately 950 hectares. The major production areas are along the Murray River, between Swan Hill in Victoria, and Waikerie in South Australia. A central commercial processing facility is located at Robinvale, Victoria.

The success of the industry is dependent on production of quality produce for export and domestic markets. Most pistachios are sold in-shell; therefore, a critical quality parameter are shells that are light in colour and free from damage and staining. In 2017, there was an increase in the incidence of dark staining on shells, with some orchards having up to 50% of nuts affected. Laboratory tests showed a range of fungi associated with this dark staining, including *Aspergillus*, *Fusarium*, *Cladosporium*, *Alternaria*, *Penicillium, Rhizopus* and *Phomopsis*. However, as most of these fungi are also effective saprophytes, it was unclear whether they were the cause of the staining or simply hitchhikers on damaged shells.

The aim of the project was to investigate the causal organism associated with dark staining, evaluate when pistachio is most susceptible to infection and elucidate other factors associated with the incidence of dark staining.

Investigations showed that levels of dark staining increased with time, and the longer the nuts remained on the tree following optimum harvest, the higher the incidence of dark stain. No correlation was found between the external hull appearance and the development of dark stain; however, it was more likely that severely damaged nuts would also have kernel damage.

In field trials with inoculation of the three main fungi identified from pistachios (*Aspergillus*, *Alternaria* and *Rhizopus*), no statistically significant correlations were found between any of the fungi and dark or light staining. It was concluded that the fungi are not the cause of dark staining but were more likely to be saprophytic on the surface of the damaged tissue.

This project did not identify the cause of dark staining of pistachio nuts; however, it did add valuable knowledge. It showed there was no correlation between external appearance of the hull and dark stain, and no correlation between any of the inoculated fungal pathogens and dark stain. The research also confirmed the dark staining became worse in the later harvest times. Therefore, with current knowledge, it is unlikely that there can be any advance warning of dark stain symptoms from the external appearance of the clusters.

Further research is needed to provide clarity on the relationships between the environmental conditions in seasons with different levels of stain, and to look at temporal and spatial variation, and physiological variation of dark staining throughout the season.

Keywords

Pistachio, dark stain, *Alternaria, Penicillium, Rhizopus*, fungal infection

Introduction

In Australia, the pistachio (*Pistachia vera* L.) industry is projected to be worth $25 million by 2020, with a current planted area of approximately 950 hectares. The major production areas are along the Murray River, between Swan Hill in Victoria, and Waikerie in South Australia. A central commercial processing facility is located at Robinvale, Victoria.

The success of the industry is dependent on production of a quality product for export and domestic markets. Most pistachios are sold in-shell, therefore shell colour is a significant parameter of quality with specifications set to be light and free of damage and staining.

Pistachios are susceptible to certain risk factors including late spring frosts, high heat events and rainfall during harvest. These risks can be negated through orchard management practices to modify tree physiology. The understanding of tree physiology and factors that influence growth and production forms the basis of successful orchard management.

In 2017, there was an increase in the incidence of dark staining on shells, with some orchards having up to 50% of nuts affected. The 2016 growing season was characterised by above average rainfall and it is known that many fungi favour wet, humid conditions for infection. Laboratory tests showed a range of fungi associated with this dark staining, including *Aspergillus spp*., *Fusarium*, *Cladosporium*, *Alternaria*, *Penicillium* and *Phomopsis*. However, as most fungi are also effective saprophytes, it was unclear whether they were the cause of the staining or simply hitchhikers on the damage.

There is limited information on the susceptibility of fruit, risk and spread of fungal infections on pistachio shells. Typically, the pistachio hull does not split before harvest, and it protects the kernel from fungal infection. In almond, hull-split is known as a high-risk period for fungal infections, when nutrient supply in the nut is high. Early split, a physiological problem in pistachios, has been correlated with a high incidence of fungal infection and is dependent on the time splitting occurs prior to harvest (Doster *et al.,* 1995). However, industry advice indicates that splitting is typically less than 2%, and the subsequent staining is on the suture side of the nut, unlike the dark stain seen in 2017/2018.

There is a need to gain information on the cause of dark staining, whether these fungal pathogens are responsible, and how they can be monitored to minimise the risk to pistachio production.

The aim of the project was to investigate the causal organism associated with dark staining, evaluate when pistachio is most susceptible to infection and other factors associated with the incidence of dark staining. The information will provide a monitoring tool for growers to use in evaluating the risk of fungal disease and assist in the future development of management strategies.

Methodology

In consultation with the research Committee of the Pistachio Industry Growers Association (PGIA), an initial project plan was established, and amended as required in response to results for the investigations. The research included the following key objectives:

- Determine the cause of dark staining incidence and the presence of fungal organisms.
- Assess the influence of environmental and plant conditions on the incidence of dark staining.
- Investigate the relationship between fungi and dark staining.

1. Determine the cause of dark staining incidence and the presence of fungal organisms.

Two field sites were established on pistachio cv. Siora at Robinvale, Victoria, and Kyalite, NSW, where high levels of dark staining had occurred in previous years. During the growing season, pistachio crops were monitored for signs of fungal disease on leaves and developing fruit.

a. **The incidence of dark staining:** was obtained from commercial hulling data in the 2017, 2018 and 2019 harvests. Nuts were graded according to existing industry standards, including light staining and dark staining.

b. **The correlation between external fruit necrosis and dark staining:** was evaluated by tagging damaged fruit and assessing staining at harvest. The possible cause of the damage was also investigated.

c. **Development and timing of dark staining**: evaluated in 2018 by collecting fruit from the two sites at 6-8 times from closed hull to second harvest. Five clusters were collected from five trees (250 fruit) at each time, or 250 shells collected from harvested fruit, and assessed for presence of dark staining. In 2019, instead of field collections, 21 lots of nuts were collected from the processing plant at Robinvale after hulling, commencing at the start of harvest 1st March 2019.

d. **Histological studies**: Scanning electron microscopy (SEM) was used to detect the presence of fungi in association with the dark stained area of nuts by photographing stained and non-stained areas of the same nuts.

e. **Isolation of fungi**: was undertaken on any fruit with disease symptoms during growth (2018), and on hull and shell material from both symptomatic (2018, 2019) and non-symptomatic (2018) nuts. Cultures were made using standard fungal isolation techniques, and fruit were also incubated in high humidity to assess natural development of fungi. Healthy nuts were inoculated *in vitro* with the fungi isolated to determine whether dark staining would result.

f. **Analysis of commercial harvest data at 2019/20 field site:** data provided by the grower from the hulling facility were analysed to determine if there were any correlations between dark staining and any of the hull characteristics recorded (e.g., hull adhering, narrow split, closed shell) and the days from harvest.

2. Assess the influence of environmental and plant conditions on the incidence of dark staining.

a. **Analysis of weather data:** Temperature, relative humidity and rainfall data was collected for the two field sites in 2017, 2018 and 2019. In the 2020 season, data was collected for the three to four months prior to harvest. All weather data was evaluated to draw any correlation with the potential for fungal infection.

b. **Analysis of polyphenol oxidase (PPO):** to determine whether enzymatic browning was involved in the dark staining, 30 nuts with various levels of stain were tested for the level of PPO activity.

3. **Investigate the relationship between fungi and dark staining.**

   a. ***In vivo* inoculations:** closed and open clusters on cv. Siora trees at a commercial planting in Peebinga, SA, were inoculated with the three main fungi recovered from pistachio hulls (*Alternaria, Rhizopus* and *Penicillium*) at 10-15 days prior to the expected harvest. The treatments were applied to both open and closed clusters. Twenty-five replicate clusters with 25-40 fruit per cluster were tagged for each of the treatments. Solutions with at least 10^5 spores/ml of each of the three fungi were sprayed on the clusters until wet and bagged in plastic bags overnight. Untreated clusters and water sprayed, and bagged clusters were used as controls. Clusters were covered with mesh bags to collect fruit when harvested. Trees were machine harvested by the grower and bags containing sample fruit were removed and collected for assessment in the laboratory. Just prior to the second harvest the remaining tagged clusters were removed and returned to the laboratory for assessment.

   b. **Effect of fungal inoculations on stain development:** At the two harvest times, inoculated and control (un-inoculated) nuts were collected and assessed for staining. From the first harvest, 25 nuts per cluster were selected at random for assessment. Of the remaining clusters collected at second harvest, the rachis was shaken and only nuts that had dropped from the rachis were assessed, as those still attached were considered immature and unable to be hulled. For both harvests, nuts were first separated into three categories according to their external appearance (clean, slight damage, significant damage), then hand hulled and separated into four categories (clean, slight grey stain at attachment end, <50% grey discoloration and >50% dark & grey stain). After discussions with the industry, the grey discoloration was considered not critical, and nuts in the >50% dark & grey stain category were then further separated into three categories (grey discoloration, light stain and dark stain). These nuts were incubated in near 100% RH to assess the development of any fungi. The three grey discoloration categories (slight gray discoloration at attachment end, <50% grey discoloration and >50% grey stain) were combined, and the final four categories analysed were clean, grey discoloration, light stain and dark stain.

Outputs

Presentations were made to the PGAI.

- February 12, 2018: Presentation by Dr Rawnsley to the PGAI board members at Mildura, Victoria, outlining the proposed research plan and methodologies. Approximately 16 people were in attendance. (Appendix i)
- September 13, 2018: Presentation by Dr Rawnsley to the Pistachio Growers Association Spring Symposium and AGM in Robinvale, Victoria. The results of project PS 16002: Pathogens and other factors contributing to dark staining on pistachio shells were presented to 55 members and industry personnel in attendance. (Appendix ii).
- 2019 research Committee meetings: Dr Rawnsley presented an update on the project findings at three Pistachio Research Committee meetings held on 2 May in Adelaide, SA; 7 June and 10 September in Mildura, Victoria. (Appendix iii).
- September 11, 2019: Presentation by Dr Rawnsley to the Pistachio Growers Association Spring Symposium and AGM at Mildura, Victoria. A research update was presented to 35 growers and industry personnel. (Appendix iv).
- February 4, 2020: a report was tabled to PGAI R&D committee, with Barbara Hall, Richard Porter and Bethany Greatz supporting the report via video conference.
- June 15, 2020: Barbara Hall, Bethany Greatz and Richard Porter presented an update on the project findings at the Pistachio Research Committee meetings via videoconference. (Appendix v).
- Sept 29, 2020: Barbara Hall, Bethany Greatz and Richard Porter presented an update on the project findings at the Pistachio Research Committee meetings via videoconference.
- Sept 30, 2020: Barbara Hall, Bethany Greatz and Richard Porter presented an update on the project findings at the 2020 Pistachio grower's symposium via videoconference.

A poster was presented by Dr Belinda Rawnsley in November 2019 at the Australian Plant Pathology Society Biennial conference. "Are pathogens responsible for dark staining of pistachio shells?" (Appendix vi).

An article on the project was submitted on 13th November to the Australian NutGrower magazine for publication. "The rise and fall of dark stained pistachio shells". (Appendix vii).

An article is in preparation for the Australian Nutgrower magazine on the results of the 2019/20 field trial.

Outcomes

1. **Determine the cause of dark staining incidence and the presence of fungal organisms.**

    a. **The incidence of dark staining:** Hulling data from 2017 showed a high incidence of dark stain, however in 2018 the incidence was much lower. In both seasons dark staining increased as the harvest season progressed (Appendices ii, iii: PGAI progress report 2018, 2019).

    b. **The correlation between external fruit necrosis and dark staining:** There was no evidence to correlate necrosis or external blackening on fruit during the growing season with development of dark staining of shells at harvest. Fruit samples collected prior to maturity did not develop dark staining on the developing embryo or shell. Dieback of clusters observed on trees was not related to dark staining of shells. In most cases, *Alternaria* was detected from clusters showing characteristic disease symptoms, although *Botryosphaeria* is commonly associated with dieback. Insects were also a possible cause of the external damage of the hull but insect damage was not related to dark staining of shells. Severe hull damage resulted in fruit deterioration and often did not develop to maturity. Dark staining occurred at hull split and hull slip and fungal infection was also more likely at fruit maturity (Appendices ii, ix: PGAI progress report 2018, M/S 103).

    c. **Development and timing of dark staining**: Staining is highly correlated with ripening and time until harvest. Dark staining occurred when the hull separated from the shell. Prior to maturity, the hull adhered to the shell and in all immature samples, dark staining was not detected. Sampling of fruit prior to harvest, at optimal harvest and after harvest showed dark staining occurred 10-12 days prior to optimal harvest and increased with time. Examination of hulled shells from multiple sites in 2018 also showed the higher prevalence of staining in nuts at the end of the season (Appendices ii, iv, ix, x: PGAI progress reports 2018, 2019; M/S 103, 104).

    d. **Histological studies**: The area of dark staining showed degradation of the surface tissue compared to the “clean” unstained area. No or minimal evidence of fungi was detected, and no fungi were present in the tissue. For this reason, staining was considered physiological, rather than a result of fungal infection (Appendices iv, v: PGAI progress reports 2019, 2020).

    e. **Isolation of fungi**: *Alternaria, Fusarium, Rhizopus* and *Penicillium* were isolated from both clean and stained shells. *Botryosphaeria* and *Alternaria* were commonly isolated from external fruit damage. Pathogenicity tests with *Alternaria* and *Penicillium* did not result in dark staining as identified by industry standards but would be classified as mould (Appendices ii, iv, ix, x, xi: PGAI progress reports 2018, 2019; M/S 103, 104, 106).

    f. **Analysis of commercial harvest data at 2019/20 field site:** There was a weak correlation between the date of harvest and the presence of dark or light stain, in concordance with previous findings of the level of stain increasing with time. However, there was no correlation between the stain levels and any of the other hull parameters measured at the hulling facility (Appendix v: PGAI progress report 2020).

2. **Assess the influence of environmental and plant conditions on the incidence of dark staining.**

    a. **Analysis of weather data:** There was no weather event in any of the years examined during this study that could be considered highly conducive to fungal infections at harvest. While there was large variability in rainfall between the sites examined, and there was more rain in January to March 2017 than in other years, there were very few high humidity days recorded (Appendices iii, v: PGAI progress reports 2019, 2020).

    b. **Analysis of polyphenol oxidase (PPO):** It is unlikely that PPOs are a cause of dark staining in

pistachios. PPO was not related to levels of staining. The enzyme was detected in dark stained shells regardless of severity, and also in light stained and clean shells. There was no difference in PPO levels from fruit collected directly from the tree or post hulling (Appendices iii, xi: PGAI progress report 2019, M/S 106).

3. **Investigate the relationship between fungi and dark staining**. No statistically significant correlations were found between any of the inoculated fungi and the development of dark or light staining. It was concluded that the fungi are not the cause of the dark stain but are saprophytic on the surface of the damaged tissue (Appendices v, xii: PGAI progress report 2020, M/S 107).

   Trends observed from the field inoculation results showed:

   - The second harvest had higher levels of both dark and light staining. This concurs with the previous results that showed higher staining later in the harvest period. Conversely, the grey discoloration observed was much lower at the second pick.
   - There was a slightly lower level of dark and light stain in the open clusters compared to closed, tight clusters.
   - No differences were observed between any of the fungal inoculations and the bagged un-inoculated control clusters in any of the four categories at the first pick. However, in the second pick there was an increase in the grey discoloration following inoculation with all three fungi.
   - Clusters inoculated with *Rhizopus* showed a decrease in both light and dark stain in the second pick, and with *Penicillium* a decrease in the incidence (or severity) of dark stain.
   - Comparing the influence of the three fungi within the three staining categories, indicated a higher level of *Rhizopus* associated with the grey discolored hulls, *Alternaria* with the light stain and *Penicillium* with the dark stain.
   - There was a trend for the *Rhizopus* and *Penicillium* to be detected at higher levels in the incubated nuts from treatments inoculated with those two fungi, but this had no impact on the level of stain detected.
   - Following incubation, more *Alternaria* was detected in the two controls and the *Alternaria* inoculated treatments than in the treatments inoculated with either *Rhizopus* or *Penicillium.*

Monitoring and evaluation

Key Evaluation Questions and associated responses.

1. To what extent has the project achieved its expected outcomes?
    a. To what extent has the project improved knowledge and awareness of dark staining?
    b. Are fungal pathogens associated with dark staining or are there other factors that require further research?

    The project has confirmed that the cause of dark staining is not directly related to fungal infection of nuts, and the fungi commonly detected in stained pistachio nuts are more likely to be saprophytes on the damaged tissue. The project findings have confirmed there is a relationship between levels of dark staining and time of harvest, with the incidence of staining more prevalent later in the season.

2. How relevant was the project to the needs of intended beneficiaries?
    a. To what extent have the project findings assisted the Pistachio industry and will the findings be adopted?

    The results of the project have clarified that fungal pathogens are not the cause of dark staining in pistachios. This has assisted the industry with their control procedures during harvest and negated subsequent unnecessary fungicide treatments. Other findings have provided important information on managing the timing of harvest to minimise the time fruit remains on the tree.

3. How well have intended beneficiaries been engaged in the project?
    a. Have regular project updates been provided to the research chair and other members of the Pistachio Growers Association?

    Updates were provided to the PGAI at the annual symposiums, and at other times as requested to the research group. The committee and grower members were kept up to date on the progression of the project findings and outcomes of seasonal field trials.

4. To what extent were engagement processes appropriate to the target audience of the project?
    a. Were project outcomes communicated to the industry in the way of presentations or publications?
    b. Were project milestones completed in the designated time frame?

    As well as presenting and engaging with PGAI committee and members at the annual symposium, the project delivered timely annual reports to PGAI. Project findings were disseminated through two articles published in the Australian Nutgrower industry magazine, and research results were communicated to the scientific community at the Australian Plant Pathology Society Biennial conference in 2019. Research was conducted in a timely manner to coincide with seasonal and physiological conditions and project milestones were completed at the designated times.

5. What efforts did the project make to improve efficiency?
    a. Were the project activities and resources appropriate to gain an understanding of dark staining?

    The project research plan and methodology ensured appropriate studies were undertaken to gain new knowledge and a greater understanding of dark staining in pistachio. Although fungal pathogens were not correlated to the incidence of dark staining, the study provided a clearer picture on the timing of occurrence and likely relationship with physiological age of the fruit on the tree and timing of harvest.

6. Has the project provided clear guidance to the industry?
    a. Has the project identified if further research is required to modify orchard management practices?

    The project has shown potential avenues for further research, including environmental and physiological conditions, which will be necessary before changes to production methods can be made to reduce the incidence of dark staining.

Recommendations

This project confirmed that the fungal pathogens detected on the stained nuts are not the cause of dark staining of pistachio nuts and provides valuable knowledge in showing there was no correlation between external appearance and the incidence of dark stain. Inoculation studies confirmed there was no correlation between any of the inoculated pathogens (*Alternaria, Rhizopus* and *Penicillium*) and dark stain. It also confirmed that the dark staining became worse in the later harvest times and the time fruit remained on the tree.

With this updated knowledge, it is unlikely there can be any advanced warning of dark stain issues from monitoring the external appearance of the clusters. However, the results conclude nuts are more at risk of dark staining if harvest is delayed or prolonged later in the season.

Further research is needed to provide clarity on the relationships between environmental conditions and dark staining in unfavourable seasons, and to consider physiological variation of fruit development during those seasons.

Suggestions include:

- Specialist climatic expertise to evaluate climate data between “good” (low stain) and “bad” (high stain) years to determine potential differences and/or triggers for dark staining.
- Determine if there is any temporal and/or spatial pattern to dark staining within the tree canopy and/or orchard to determine potential differences and/or triggers for dark staining.
- Further evaluate time of harvest and physiological stages in relation to dark stain development.

References

Araji, A., Grammar, T. A., Gertzen, R., Anderson, S. D., Mikulic-Petkovsek, M. Veberic, R., Phu, M. L., Solar, A., Leslie, C. A., Dandekar, A. M., Escobar, M.A. (2014). Novel Roles for the Polyphenol Oxidase Enzyme in Secondary Metabolism and the Regulation of Cell Death in Walnut. *Plant Physiology*. 164. 1191-1203. https://doi.org/10.1104/pp.113.228593

Baggio, J.S., Gonçalves, F.P., Lourenço, S.A., Tanaka, F.A.O., Pascholati, S.F. and Amorim, L. (2016), Direct penetration of *Rhizopus stolonifera* into stone fruits causing rhizopus rot. *Plant Pathology*, 65: 633-642. doi:10.1111/ppa.12434)

Boedo, Cora, Le Clerc, Valérie, Briard, Mathilde, Simoneau, Philippe, Chevalier, Michel, Georgeault, Sonia & Poupard, Pascal. (2008). Impact of carrot resistance on development of the Alternaria leaf blight pathogen (*Alternaria dauci*). *European Journal of Plant Pathology*. 121. 55-66. 10.1007/s10658-007-9241-6.)

Doster, M. A., Michailides, T. J. (1995). The Relationship between Date of Hull Splitting and Decay of Pistachio Nuts by *Aspergillus* Species. *Plant Dis.* 79:766-769

Doster, M. A., Michailides, T. J. (1997). The effect of delaying harvest on pistachio nut quality. California Pistachio Industry Annual Rep Crop Year 1996–97. *Fresno*, CA. p 82–4.

Doster, M. A., Michailides, T. J. (1999). Relationship between shell discoloration of pistachio nuts and incidence of fungal decay and insect infestation. *Plant Dis*. 83(3):259-264. doi: 10.1094/PDIS.1999.83.3.259. PMID: 30845504.

Goldhamer, D.A., Michailides, T.J., Morgan, D.P. (2002). Buried drip irrigation reduces fungal disease in pistachio orchards. *California Agriculture* (56) 4. DOI: 10.3733/ca.v056n04p133

Donis-González, E. R., Guyer, D. E., Fulbright, D. W. (2016). Quantification and identification of microorganisms found on shell and kernel of fresh edible chestnuts in Michigan. *J Sci Food Agric*. 96: 4514-4522. https://doi.org/10.1002/jsfa.7667

Khosravi, A. R., Shokri, H., Ziglari, T. (2007). Evaluation of Fungal Flora in Some Important Nut Products (Pistachio, Peanut, Hazelnut and Almond) in Tehran, Iran. *Pakistan Journal of Nutrition*, 6: 460-462.

Lichtemberg P, Puckett R, Felts D, Luo Y, Doster L, Rodriguez D, Michailides T. (2018). Survey of the pathogen of Alternaria late blight reveals different levels of carboxamide fungicide resistance in the main pistachio producing regions of California. *Calif Agr* 72(3):170-178. https://doi.org/10.3733/ca.2018a0022

Pleysier, C.E., Bayliss, K.L., Dell, B. *et al*. Temperature, humidity, wounding and leaf age influence the development of *Alternaria alternata* lesions on leaves of *Paulownia fortunei*. *Australasian Plant Pathology* 35, 329–333 (2006). https://doi.org/10.1071/AP06030

Qian, X., Yang, Q., Zhang, Q., Abdelhai, M. H., Dhanasekaran, S., Serwah, B., Gu, N., & Zhang, H. (2019). Elucidation of the Initial Growth Process and the Infection Mechanism of *Penicillium digitatum* on Postharvest Citrus (Citrus reticulate Blanco). *Microorganisms*. 7(11), 485. https://doi.org/10.3390/microorganisms7110485

Thomson, S. V., Mehdy, M. C. (1978). Occurrence of *Aspergillus flavus* in pistachio nuts prior to harvest. *Phytopathology*. 68:1112-1114. DOI: 10.1094/Phyto-68-1112

Zarei, M., Davarynejad, G., Abedi, B., Kafi, M. Biabani, A. (2014). Changes in Physical Properties, Chemical Composition and Antioxidant Activity of Four Pistachio Cultivars at Ten Maturity Stages. *Advances in Environmental Biology*. 8(10):106-115.

Intellectual property, commercialisation and confidentiality

No project IP, project outputs, commercialisation or confidentiality issues to report.

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PPO (Poly Phenol Oxidase) analysis was completed by SARDI.

Appendices

Appendix i

Pistachio PPT PS16002 Presentation to PGAI Board 12 Feb 2018

Appendix ii

Pistachio PPT PS16002 Presentation to PGAI Spring Symposium & AGM 13 September 2018

Appendix iii

Pistachio PPT PS16002 Presentation to PGAI Research Committee 2019

Appendix iv

Pistachio PPT PS16002 Presentation to PGAI Spring Symposium & AGM 11 September 2019

Appendix v

Pistachio PPT PS16002 Presentation to PGAI Research Committee February 4 2020

Appendix vi

Pistachio PPT PS16002 Presentation to PGAI Research Committee June 15 2020

Appendix vii

Pistachio PPT PS16002 Presentation to PGAI Research Committee Sept 29 2020

Appendix viii

Pistachio PPT PS16002 Presentation to PGAI Spring Symposium & AGM 30 September 2020

Appendix ix

Poster presentation 'Are pathogens responsible for dark staining of pistachio shells' B. Rawnsley

Appendix x

Article for Australian NutGrower magazine Nov 2019

Appendix xi

PGAI progress report 2018

Appendix xii: Hort Innovation Milestone 102

Appendix xii: Hort Innovation Milestone 103

Appendix xiv: Hort Innovation Milestone 104

Appendix xv: PGAI progress report 2019

Appendix xvi: Hort Innovation Milestone 106

Appendix xvii: PGAI 6-month milestone progress report 2020 107

Appendix xviii: Hort Innovation Milestone 10

Appendix i: Pistachio PPT PS16002 Presentation to PGAI Board 12 Feb 2018

Pathogens and other factors contributing to dark staining in pistachio

Hort Innovation Project Code PS16002

Dr Belinda (Brandy) Rawnsley
AgXtra

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AgXtra

- Formed in 2003 (AgXtra as of Oct 2016)
- Based in Newton, Adelaide
- 15 staff
- Conduct independent agricultural research for our clients
- Plant pathologist – previously with SARDI Horticulture Diagnostics, Waite Institute
- Lab facilities for fungal culturing

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Objectives

- Determine the causal organism associated with dark staining
- Evaluate when pistachio is most susceptible to infection and factors affecting the incidence of infection by the causal agent(s).
- Develop guidelines to assist monitoring and risk of dark staining on pistachio in the field.
- Assess the incidence and development of dark staining in shelled nuts pre-harvest and post-harvest

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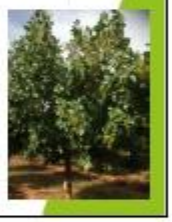
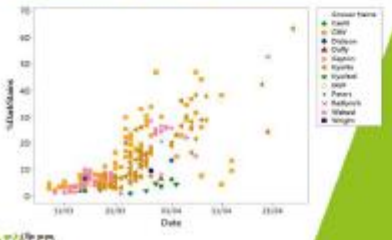
Project Outline

- Project commenced 25 Sept 2017
- Field and lab-based research
- Two trial sites
  - CMV Farms, Robinvale, VIC
  - Kyalite Pistachios, Kyalite, NSW
- Data collated from previous seasons



4

Dark staining 2017 Harvest data



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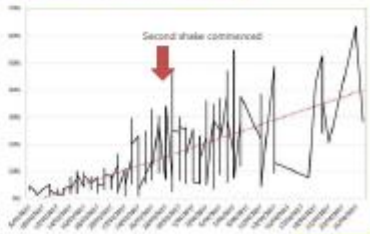
Progression of dark staining



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Trial methods

When does dark staining occur?

- Site visits each month
- Monitoring fruit for dark staining

Effect and cause of dark staining?

- Five trees per site
- Collect 5 clusters per tree every 5 days:
  - -15,-10,-5 days pre-harvest
  - Optimum harvest date
  - +5, +10, +15, +20, +25,+30 post-harvest
  - Assess severity and incidence

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Results

- Limited staining on developing fruit during the season
- Symptoms can be maintained at 4°C
- Many symptoms attributed to insect stings
- Do not progress
- Superficial surface blackening that does not penetrate embryo
- *Alternaria* isolated

AgXtra

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Results

- Cluster dieback
- Remained isolated on cluster
- Either whole cluster died or no further progression with desiccation of affected part
- *Alternaria* isolated
- Also confirmed by Barb Hall (SARDI)

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Pathogenicity studies

- Alternaria Late Blight causes circular lesions on leaves – this was not observed
- Can cause dark staining
- Need to confirm if *Alternaria* is causal agent or secondary
- Is *Alternaria* detected during harvest period or other pathogens?
- Infect green tissue to replicate symptoms
- Delayed hull on shell post-optimum harvest date

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11

Appendix ii: Pistachio PPT PS16002 Presentation to PGAI Spring Symposium & AGM 13 Sept 2018

Pathogens and other factors contributing to dark staining in pistachio

Hort Innovation Project Code
PS16002

Dr Belinda (Brandy) Rawnsley
AgXtra

AgXtra

1

Objectives

- Evaluate the occurrence and incidence of dark staining and when pistachio is most susceptible
- Determine the causal organism associated with dark staining
- Assess the factors associated with the incidence of infection by the causal agent(s)
- Develop guidelines to assist monitoring and risk of dark staining on pistachio

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2

Project Outline

- Project commenced 25 Sept 2017
- Field and lab-based research
- Two trial sites
  - Site 1, Robinvale, VIC
  - Site 2, Kyalite, NSW
- Data collated from previous seasons
- New data generated to target objectives

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Progression of dark staining 2017 Hulling data

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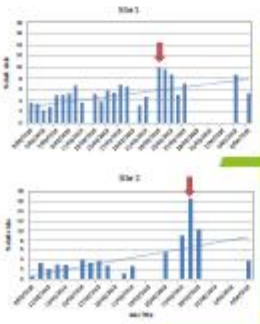
4

Progression of dark staining 2018 Hulling data



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Climatic differences 2017 and 2018

Monthly Rainfall (mm)

Site 1	Year	Jan	Feb	March
	2017	23.9	6.4	13.0
	2018	5.0	39.2	0.4

Site 2	Year	Jan	Feb	March
	2017	6.0	10.4	24.4
	2018	19.0	0.0	0.0

Site 1 - isolated rainfall events
2017
20 Jan 14.0 mm
22 March 11.8 mm
2018
12 Feb 28.6 mm

Site 2 - isolated rainfall event
2017
22 March 22.0mm

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Two main questions for the first year research

1.When does dark staining occur
- Can DS be detected in the field prior to harvest?
- Are there symptoms on trees to identify risk?
- Can DS be detected early?

2. What causes dark staining?
- Are fungal pathogens responsible?
- If so, what is the casual agent?

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Trial methods

1. When does dark staining occur?
- Site visits each month from fruit set
- Monitoring fruit for dark staining
- Five trees per site
- Collect 5 clusters per tree every 5 days and sampled 50 fruit:
  - -15,-10,-5 days pre-harvest
  - Optimum harvest date
  - +5, +10, +15 post-harvest until 2nd shake
  - Assessed incidence of DS at each timing

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Field observations

- During the growing season, looked for trees with signs of fungal infection.
- Alternaria has been associated with shell staining:
  - symptoms appear on leaves mid summer
  - on mature fruit, hull lesions are black
  - Favoured by high relative humidity.
  - Aging leaves and fruit more susceptible.
  - More severe on fruit bearing shoots.
- Mostly healthy but dieback observed

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Dieback was detected on isolated trees

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Alternaria – isolated from clusters with dieback

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Results

- Cluster dieback
- Remained isolated on cluster
- Either whole cluster died or no further progression with desiccation of affected part
- *Alternaria* isolated
- Also confirmed by Barb Hall (SARDI)
- No association with DS

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Storage of fruit pre-harvest

- Fruit clusters were stored at 4°C at each collection period
- Fruit remained viable for up to 4 weeks prior to hull slip
- Yet rapidly deteriorated after hull slip
- Moisture between the hull and the shell resulted in fast decay and increased incidence of dark staining

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-19 DBH
13/2/18

0 DBH
4/3/18

+17 DBH
21/3/18

Fruit clusters collected at number of days before harvest (DBH)

Site 1 – 1st shake 4/3/18; 2nd shake 21/3/18
Site 2 – 1st shake 17/3/18; 2nd shake 28/3/18

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Removal of nut from hull

- Hull adheres to shell prior to maturity
- Prior to this stage, the nut could not be removed from the hull
- No dark staining prior to hull slip

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Dark staining apparent at hull slip

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Dark stained shell from stained hull

- In some cases, outside hull showed no blackening
- Blackening observed inside hull
- Indicates may start internally – not externally

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Severe dark stained hull > Dark stained shell

- Severe blackening of hull usually associated with fungal infection
- In many cases, nut was not viable

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Incidence of dark staining

-9 DBH DS detected

-11 DBH DS detected

site 1		site 2	
Date	DS (%)	Date	DS (%)
7/02/2018	0	7/02/2018	0
14/02/2018	0	21/02/2018	0
19/02/2018	0	28/02/2018	0
26/02/2018	0.4	6/03/2018	0.8
28/02/2018	2.0	14/03/2018	4.0
5/03/2018	2.8	26/03/2018	12.8*
15/03/2018	4.8		
21/03/2018	8.0		
1st shake	4/04/2018	1st shake	17/04/2018
2nd shake	21/04/2018	2nd shake	28/04/2018

*one fruit cluster had 80% infected fruit, all husks severely split and skin exposed

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Results

- Limited staining on developing fruit during the season
- Many external symptoms attributed to insect stings
- Superficial surface blackening that did not penetrate embryo
- *Alternaria, Fusarium* and *Rhizopus* were isolated from clean and DS shells

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Causes of dark staining?

- Cultures made of fungi isolated from shells
- Cultures of clean shells and DS shells compared

Koch's postulates :

1. The microorganism must be found in abundance in all others showing symptoms, but should not be found in healthy organisms.
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The cultured microorganism should cause disease when introduced into a healthy organism.
4. The microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

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Clean unstained shells – natural infection Rhizopus

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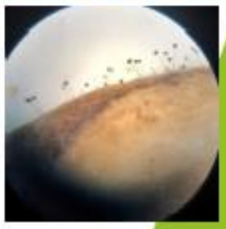
Dark stained shells – also had infection by Rhizopus



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Alternaria – isolated from pistachio and inoculated shells (Koch's)

- Segments of clean and DS shells placed on potato dextrose agar (PDA) to isolate fungi
- Spores identified
- New cultures made and shells placed on fungus
- Shells removed and development of DS recorded

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Natural dark staining vs dark staining from inoculation

Natural dark staining

Fungal inoculated dark staining

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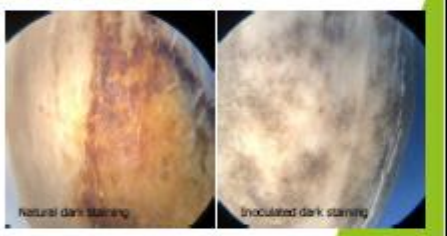
Close up of dark staining



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Research Summary

- Dark staining occurs at hull slip, not detected prior
- Occurs approx. 10 days before optimum harvest
- Crop physiology aligns with the development of DS at harvest time
- Prolonged exposure to air and moisture increases risk of dark staining
- Unlikely to be fungal pathogen, symptoms of DS could not be replicated

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Phenolics

- Enzymatic reaction that causes browning
- Pistachios are high in phenols
- Staining can occur as result of the breakdown of phenols in pistachio
- Oxidation of phenolics upon exposure of the shell to air and moisture = polyphenol oxidation
- Presence of oxygen causes the reaction
- Measure phenols in fruit at various maturity to assist harvest time?

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What Next?

- Investigate harvest conditions
  - Fungicides deemed unnecessary
  - Relative humidity higher at end of season
  - Variability of fruit retained on tree
  - Application of plant growth regulators to improve uniformity of maturity
  - Delays in processing
- Storage conditions
- Analyse temp and RH conditions of fruit after harvest
- Comments

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30

Appendix iii: Pistachio PPT PS16002 Presentation to PGAI Research Committee 2019

Pathogens and other factors contributing to dark staining in pistachio

Hort Innovation Project Code PS16002
Pistachio R&D Committee

Dr Belinda (Brandy) Rawnsley
June 6 2018

AgXtra

1

Objectives

- Evaluate the occurrence and incidence of dark staining and when pistachio is most susceptible
- Determine the causal organism associated with dark staining
- Assess the factors associated with the incidence of infection by the causal agent(s)
- Develop guidelines to assist monitoring and risk of dark staining on pistachio

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Objective 1. Occurrence of dark staining

1.When does dark staining occur?

- Can DS be detected in the field prior to harvest?
- Are there symptoms on trees to identify risk?
- Can DS be detected early?

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1. Dark staining occurs at feathering

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2. Dark staining occurs 10 days prior to optimum harvest

	site 1		site 2		
	Date	DS (%)		DS (%)	
	7/02/2018	0	7/02/2018	0	
	13/02/2018	0	21/02/2018	0	
	19/02/2018	0	28/02/2018	0	
	23/02/2018	0.4	6/03/2018	0.8	
-9 days before harvest (DBH) DS detected	28/02/2018	2.0	14/03/2018	4.0	-11 days before harvest (DBH) DS detected
	5/03/2018	2.8	26/03/2018	12.8*	
	15/03/2018	4.8			
	21/03/2018	8.0			
	1st shake	4/03/2018	1st shake	17/03/2018	
	2nd shake	21/03/2018	2nd shake	28/03/2018	

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Robinvale rainfall (mm) 2017 and 2018

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6

Kyalite rainfall (mm) 2017 and 2018

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3. Dark staining cannot be correlated to external blackening

- No symptoms on external fruit cluster
- Incidence increases with time on tree
- Prolonged exposure to air and moisture increases risk of dark staining

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Objective 2. What causes dark staining

- Are fungal pathogens responsible?
- If so, what is the casual agent?

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1. Fungi isolated from dark stained and unstained shells

Clean unstained shells – natural infection Rhizopus

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Dark stained shells – also had infection by Rhizopus

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Rhizopus

12

Specific isolation of DS shell after surface sterilisation

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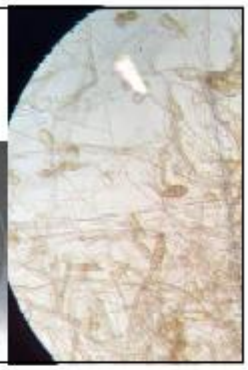
Alternaria



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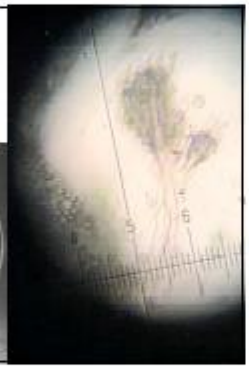
Penicillium



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Season 2018

- DS at maturity
- Follow the progression of dark staining
- We know it happens very close to harvest, but are there different fungal involved?
- Collect pistachio samples to assess temporal and spatial variability
- Collect de-hulled samples prior to heating nuts (partially processed nuts)

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Collection of DS shells following de-hulling

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Incidence (%) of fungal types at different sites

	Rhizopus	Penicillium	Alternaria	Fusarium
Lot 17	50	50	60	0
Lot 139	100	30	20	10
Lot 185	0	100	20	40
Lot 193	100	60	0	0
Lot 299	0	50	0	20
Lot 301	50	20	0	0
Lot 302	50	10	10	20
Lot 305	0	40	30	0
Lot 306	0	40	20	0
Lot 308	100	10	0	10
Unshelled	0	20	0	0
Fungal %	45	41	16	10
Unshelled %	0	20	0	0

Partially processed nuts prior to heating (n=200 total)

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Is Rhizopus and Alternaria only in dark stained shells?

Shell	Rhizopus	Alternaria
Clean shells	24%	38%
Dark stained	28%	56%

19

1. Cause of dark staining inconclusive

What we do know:

- Rhizopus, Penicillium and Alternaria are the major fungal species isolated
- BUT the fungi have been isolated from both stained and clean shells
- Cannot draw strong conclusion that staining is caused by these fungi
- To be a pathogen, needs cause symptoms

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2. Inoculation did not produce same symptoms

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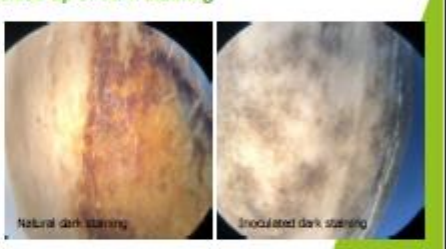
Close up of dark staining



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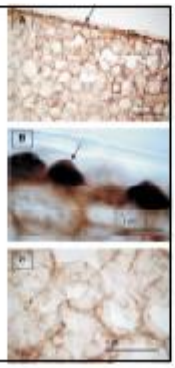
Other factors associated with staining

- Polyphenol oxidases (PPO) are released during fruit ripening
- The enzymatic reaction generates a black pigment called melanin that causes brown spots
- PPO increase with fruit maturity



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Other factors associated with staining

- The PPO reaction does not happen until oxygen is introduced = correlates with feathering and the time we see DS
- Is PPO associated with dark staining?
- Polyphenolic oxidase (PPO) analysed by SARDI Food Innovation Analytical Laboratory (West Beach)

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25

Polyphenol oxidase (PPO) enzyme results

Sample Code	severity of staining					
	FRESH	Light	<2%	2-25%	>25%	>50%
1		5.99	5.45	6.47	8.02	7.10
2		-	7.64	-	6.77	5.39
3		-	-	5.30	7.89	5.00
4		6.49	5.00	-	5.92	7.52
5		-	-	-	7.64	8.00
6		-	7.57	-	8.54	6.39
7		-	6.90	-	7.32	-
8		-	8.00	-	-	-
9		-	-	-	7.89	-
10		-	-	10.74	10.74	-
FRESH CLEAN	8.00	-			-	-
FRESH STAINED	7.99	-			-	-
Heat lot 1				7.74		
Heat lot 6		-	-	-	8.14	-

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Enzyme activity expressed as x 10^3 U mg^{-1}

26

What next?

- Histological (cellular) analysis – are there fungal strands in clean and stained nuts?

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What next?

- Histological (cellular) analysis – are there fungal strands in clean and stained nuts?
- Inoculation of fruit on trees with Rhizopus and Alternaria to replicate dark staining (in conducive artificial conditions)
- Polyphenolic oxidation – PPO can be resistant to temps over 90C. Was PPO a wound response?
- Further funding opportunity through SA Govt Innovation Connections

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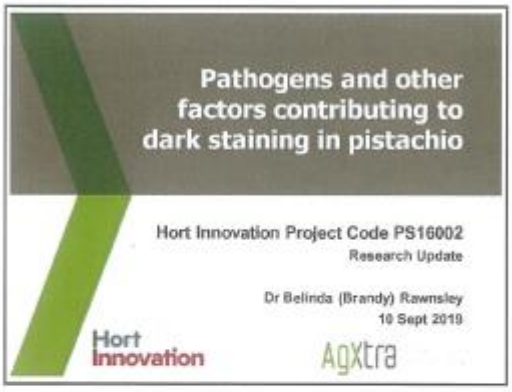
Appendix iv: Pistachio PPT PS16002 Presentation to PGAI Spring Symposium & AGM 10 Sept 2019



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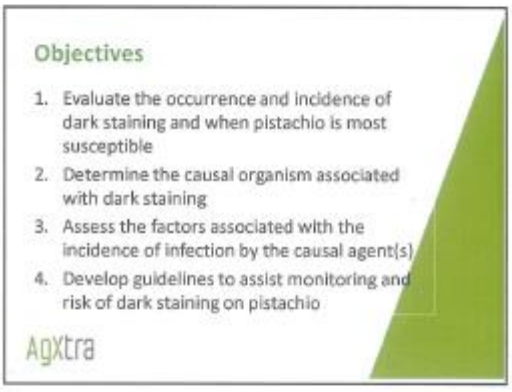

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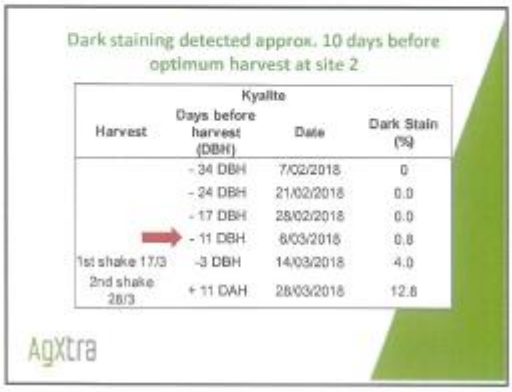

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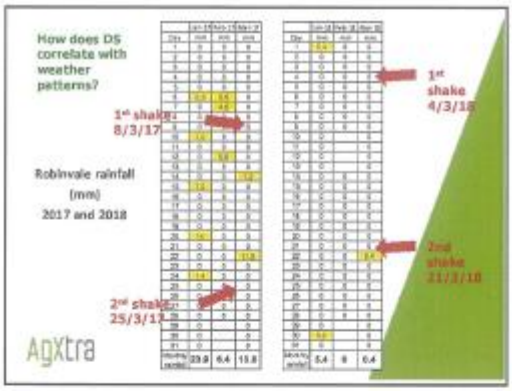
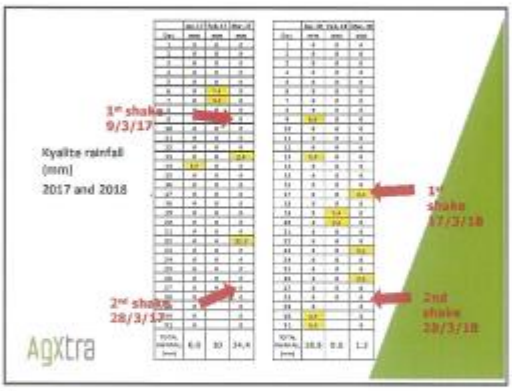

How does DS correlate with weather patterns?
1st shake 8/3/17
Robinvale rainfall (mm) 2017 and 2018
2nd shake 25/3/17
1st shake 4/3/18
2nd shake 21/3/18
AgXtra

7


1st shake 9/3/17
Kyalite rainfall (mm) 2017 and 2018
2nd shake 28/3/17
1st shake 17/3/18
2nd shake 23/3/18
AgXtra

8


Finding 3: Dark staining cannot be correlated to external blackening
No symptoms on external fruit cluster
Incidence increases with time on tree
Prolonged exposure to air and moisture increases risk of dark staining
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9


What causes dark staining
Are fungal pathogens responsible?
If so, what is the casual agent?
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Finding 4: Fungi isolated from dark stained shells
Infection by the fungus Rhizopus
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....but also fungi isolated from unstained shells
Clean unstained shells – natural infection Rhizopus
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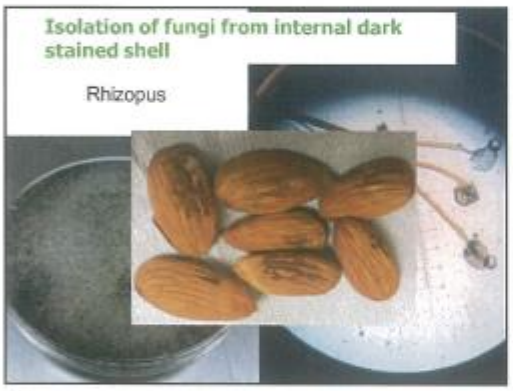

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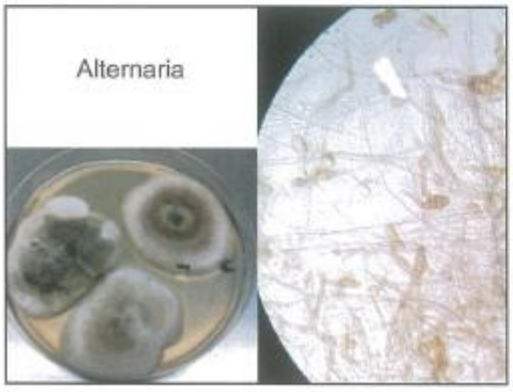

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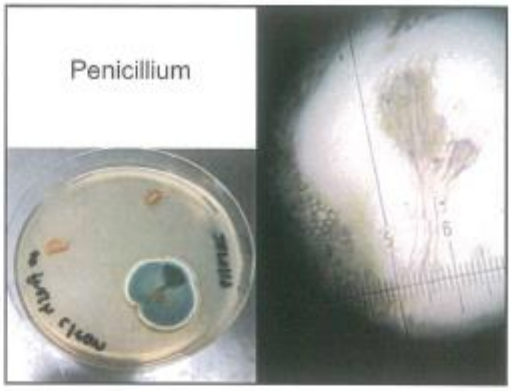

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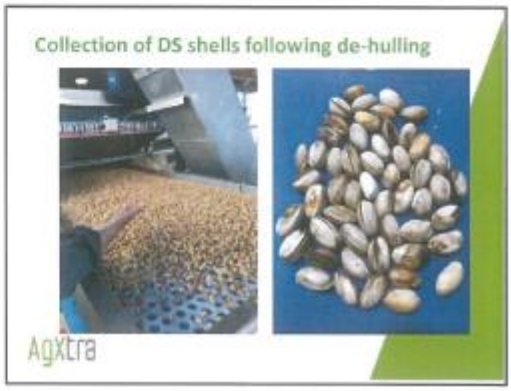

18

Incidence (%) of fungal types at different sites

	Rhizopus	Penicillium	Alternaria	Fusarium
Lot 17	50	50	60	0
Lot 139	100	30	20	10
Lot 185	0	100	20	40
Lot 193	100	60	0	0
Lot 299	0	50	0	20
Lot 301	50	20	0	0
Lot 302	50	10	10	20
Lot 306	0	40	30	0
Lot 306	0	40	20	0
Lot 308	100	10	0	10
Untained	0	20	0	0
Fungal %	45	41	16	10
Untained %	0	20	0	0

Partially processed nuts prior to shelling
n=200 total

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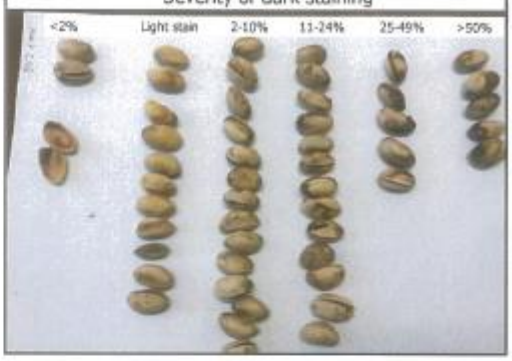
Severity of dark staining



22

Is Rhizopus and Alternaria only in dark stained shells?

Shell	Rhizopus	Alternaria
Clean shells	24%	38%
Dark stained	28%	56%

20

Polyphenol oxidase (PPO) enzyme results

Sample Code	severity of staining					
	FRESH	Light	<2%	2-25%	>25%	>50%
1		5.99	5.45	6.47	8.02	7.18
2			7.64		6.77	5.39
3				5.30	7.69	5.88
4		6.49	5.88		5.92	7.52
5					7.64	8.68
6						8.39
7						
8						
9						
10				10.74	10.74	
FRESH CLEAN	6.80					
FRESH STAINED	7.69					
Heat lot 1				7.74		
Heat lot 8					8.14	

Browning enzymes detected in ALL shells

Enzyme activity expressed as x 10^{n} U mg^{-1}

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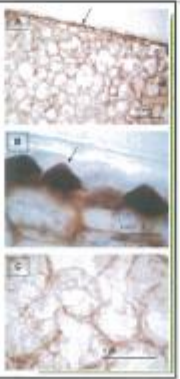
23

Other factors associated with staining

- Polyphenol oxidases (PPO) are released during fruit ripening
- The enzymatic reaction generates a black pigment called melanin that causes brown spots
- PPO increase with fruit maturity
- Happens in the presence of oxygen = hull slip



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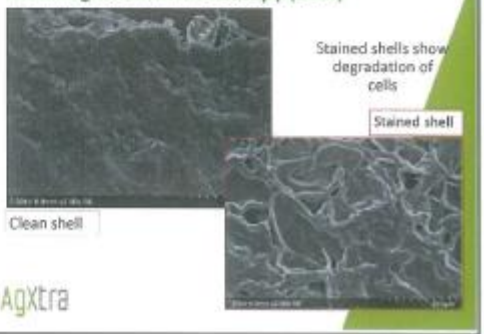
Scanning electron microscopy (SEM)



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Fungi were detected on stained shells
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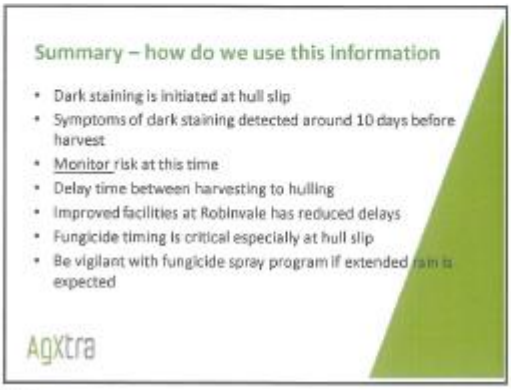
Summary – how do we use this information
• Dark staining is initiated at hull slip
• Symptoms of dark staining detected around 10 days before harvest
• Monitor risk at this time
• Delay time between harvesting to hulling
• Improved facilities at Robinvale has reduced delays
• Fungicide timing is critical especially at hull slip
• Be vigilant with fungicide spray program if extended rain is expected
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Appendix v: Pistachio PPT PS16002 Presentation to PGAI Research Committee June 15 2020

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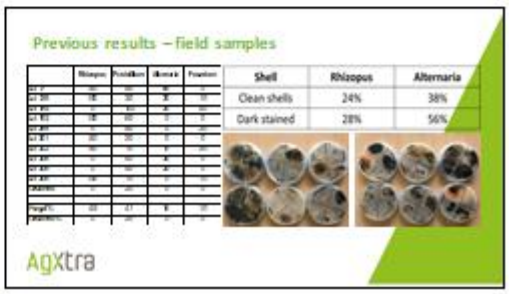

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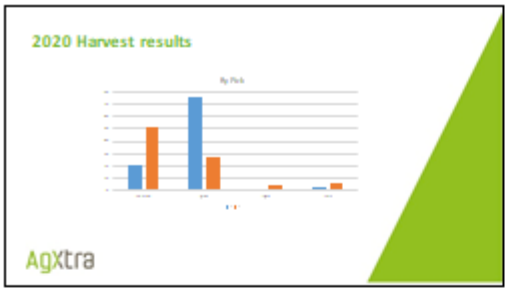

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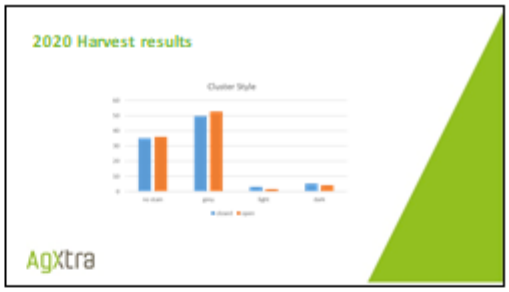

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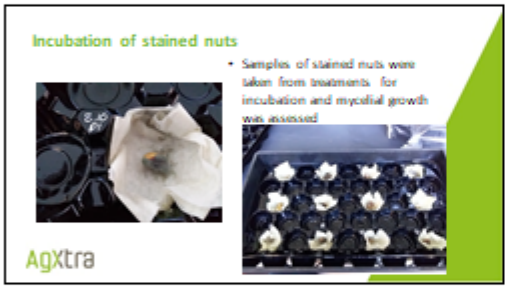

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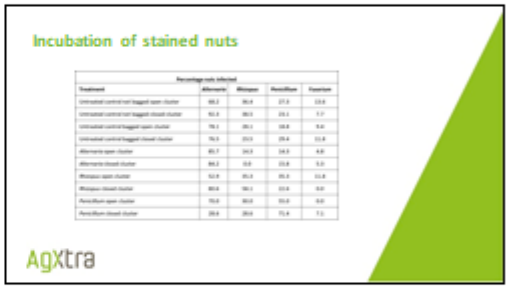

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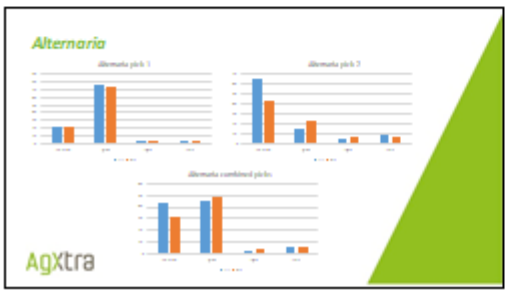

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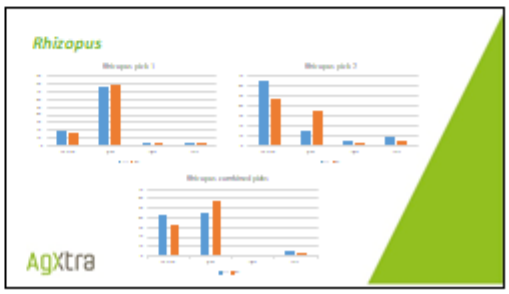

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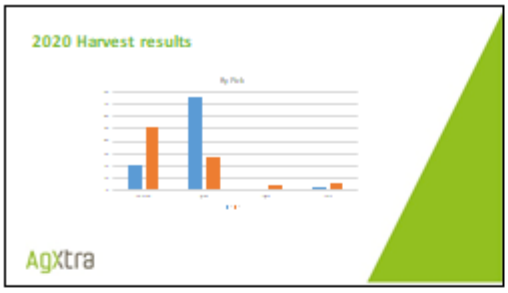

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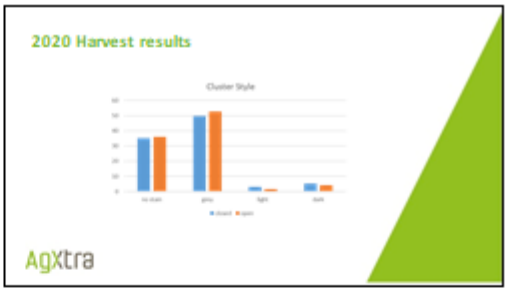

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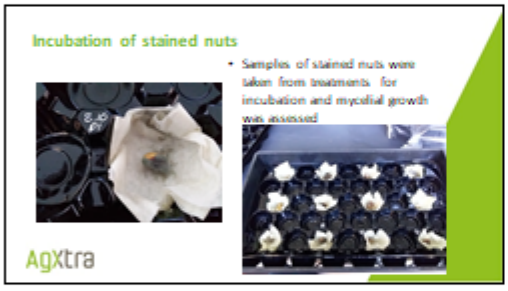

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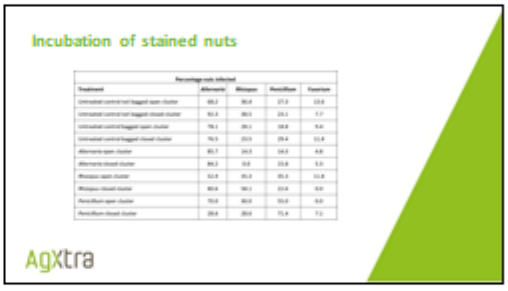

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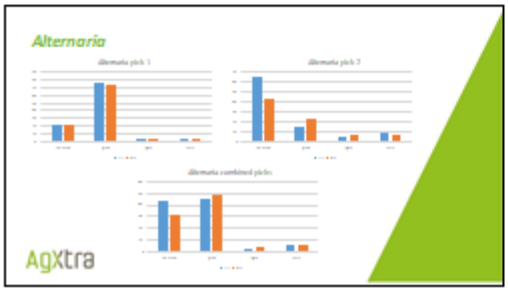

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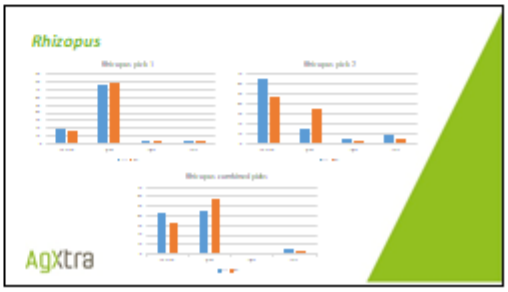

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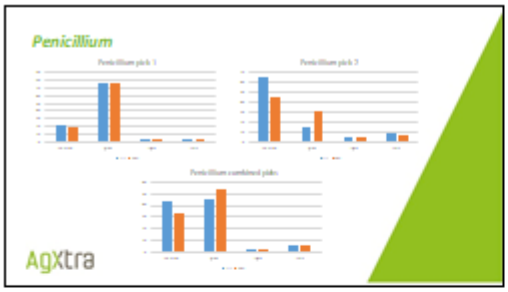
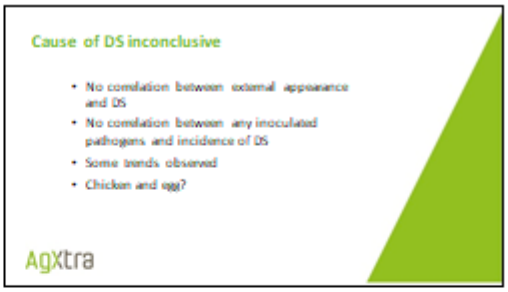

Penicillium
AgXtra

19


Cause of DS inconclusive
• No correlation between external appearance and DS
• No correlation between any inoculated pathogens and incidence of DS
• Some trends observed
• Chicken and egg?
AgXtra

20


What next?
• Physiological variation throughout season?
• Picking times?
• Environmental conditions in "bad" season?
• Tannins?
AgXtra

21

Appendix vi: Poster presentation: Are pathogens responsible for dark staining of pistachio shells b. Rawnsley

Are pathogens responsible for dark staining of pistachio shells?

Dr Belinda (Brandy) Rawnsley

brandy.rawnsley@syngenta.com

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The Australian pistachio industry is valued over $35.4 million. Most pistachios are sold in-shell, with shell colour being a significant parameter of quality. Shells are expected to be light and free of damage and staining. Dark staining of pistachio shells can cause a significant reduction in production

Aim

The project 'Pathogens and other factors contributing to dark staining on pistachio shells' (PS16002) is a strategic levy investment under the Hort Innovation Pistachio Fund to determine the causal agent and other factors associated with dark staining and when pistachio is most susceptible.

Materials and Methods

Field studies were conducted in Robinvale, VIC and Kyalite, NSW to determine the development and timing of dark staining.

Fruit were collected every 5 days during fruit ripening until optimum harvest and beyond (Fig. 1).

Fruit were examined for symptoms of dark staining of the shell (Fig. 2). Nuts were graded according to industry standards, including light staining and dark staining.

Dark stained and clean shells were surface sterilised in 0.4% NaOCl, rinsed in water placed on potato dextrose agar (PDA). Arising fungi from shells were identified using spore and morphological characteristics.

Figure 1. (a) Pistachio tree and (b) Pistachio fruit cluster near harvest

Figure 2. Dark stained pistachio shells

Results and discussion

Dark staining was first detected when the hull no longer adhered to the shell, 10 days prior to optimum harvest.

The severity of dark staining progressively worsens with ripe fruit left on the tree. Delayed harvesting increases the risk of dark staining.

Fungi were isolated from shells with and without dark staining including *Alternaria*, *Fusarium*, *Penicillium* and *Rhizopus* sp. A high proportion of dark stained shells were infected with either *Rhizopus stolonifer* or *Alternaria alternata* (28% and 56%, respectively).

Infection in clean shells may be latent and dark staining symptoms only expressed in conditions conducive for symptom expression.

Although Alternaria symptoms were not detected on leaves and fruit during the growing season, there is a strong correlation between fungal infection and dark staining. Incidence of dark staining may be very seasonal dependent, with environmental parameters such as high relative humidity at the time of hull slip necessary for fungal infection to occur

Hort Innovation AgXtra Research Partners for Crop Innovation

Appendix vii: Article for NutGrower magazine Nov 2019

The rise and fall of dark stained pistachio shells

Dr Belinda (Brandy) Rawnsley

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Since about 80% of pistachios are sold in-shell, shell colour is a significant parameter of quality. Shells are expected to be light and free of damage and staining. Dark staining of pistachio shells is correlated with poor quality nuts and can cause a significant reduction in marketable product. There are suggestions that fungal infection, insect damage and poor weather conditions contribute to dark staining yet the cause is unknown. In 2016, the industry recorded one of the lowest seasons for the incidence of dark staining. In comparison, dark staining was severe in 2017 and the industry experienced the second worst season only behind the fungal epidemic of 2011. Since then the incidence of dark staining has been low again. So why this change between seasons?

To understand what causes dark staining, a project was established by the Pistachio Growers Association Incorporated (PGAI) together with Hort Innovation (project PS16002). To determine the causal agent of dark staining, we first needed to evaluate when pistachio were most susceptible and when dark staining developed.

In California, Alternaria Blight affects leaves and fruit and is often associated with dark staining of pistachio shells. Orchards were monitored in Robinvale, VIC and Kyalite, NSW with little to no Alternaria symptoms observed during the growing season. But what about cluster dieback? Infected clusters were collected but were found to be infected with *Botryosphaeria*. Most commonly, infection resulted in death of the cluster and the fruit well before ripening.

With pistachios having uneven maturity, they are usually harvested twice, 10 to 14 days apart. Leading up to ripening, fruit was collected every 5 days until optimum harvest and beyond. Dark staining was first detected when the hull no longer adhered to the shell, 10 days prior to optimum harvest. The incidence increased after the first harvest shake. The onset of dark staining at hull slip was likely to correlate with exposure of the nut to oxygen and moisture. This is similar to disease development of *Rhizopus stolonifer* at early hull split in almond. The higher the relative humidity, the greater the incidence of infection.

In harvest data from recent years, the second shake always showed nuts with higher dark stain incidence than the first shake. Field studies also confirmed there was very low incidence of dark staining at hull slip, yet much more at the very end of the season. The severity of dark staining progressively worsens with ripe fruit left on the tree. For this reason, delayed harvesting increases the risk of dark staining.

So we know when dark staining occurs. But why? Collected nuts were graded according to industry standards, including light staining and dark staining. Fungal pathogens were isolated from shells with and without dark staining including *Alternaria*, *Fusarium, Penicillium* and *Rhizopus* sp. After surface sterilisation and laboratory isolation, a high proportion of dark stained shells were found to be infected with either *Rhizopus stolonifer* or *Alternaria alternata* (28% and 56%, respectively). Although fungi were also isolated from clean shells, this infers the fungi may have been latent – that is, dormant with conditions not conducive for symptom expression.

The findings so far indicate there is a strong correlation between fungi and dark staining. Unfortunately for research but lucky for the pistachio industry, conditions in the past year have not promoted dark staining. The incidence of dark staining may be very seasonal dependent, with environmental

parameters such as high relative humidity at the time of hull slip necessary for fungal infection to occur. The 2017 season of high dark staining was not considered wet, however relative humidity right at hull slip may have been an influencing factor in the development of dark staining.

Time of harvesting plays an important role in the incidence of dark staining. Studies conducted in California showed delaying harvest time by 10 to 20 days significantly increased the number of nuts with excessive shell stain (Doster and Michailides, 1997). However, trees are likely to retain immature fruit at the first shaking, and so an early harvest of all fruit may not be achievable. As a fresh crop, pistachios must be hulled and dried within 24 hours of harvest so there is a restricted window between harvesting and processing. Careful monitoring and prioritisation of harvest time for selected blocks will assist to reduce the incidence of pistachio with dark staining.

With the experience of wet, unfavourable conditions in 2011, growers have established fungicide programs that may successfully inhibit the development of dark staining. If environmental conditions are conducive for fungal infection, we may see a greater incidence of dark staining if fungicide sprays are not implemented in extended rain periods, especially at hull slip when dark staining is initiated. As with any season, vigilance and monitoring are the keys to successful orchard management.

Reference: Doster MA, Michailides TJ. 1997. The effect of delaying harvest on pistachio nut quality. California Pistachio Industry Annual Rep Crop Year 1996–97. Fresno, CA. p 82–4.

Appendix viii: HIA Milestone 102

Milestone Report

Project title:

Pathogens and other factors contributing to dark staining on pistachio shells

Project code:

PS16002

Milestone number:

102

Project leader:

Dr Belinda Rawnsley

Delivery partner:

AgXtra

Report author:

Dr Belinda Rawnsley

Milestone due date:

23/03/2018

Submission date:

23/03/2018

Confidentiality:

Is this report confidential?

No

Milestone description:

1. Project plan, monitoring and evaluation plan.
2. Industry monitoring tool for identification of dark staining

Milestone achievement criteria:

1. Meet with Pistachio Growers Association (PGAI) to agreed project plan
2. Monitoring and Evaluation Plan, Stakeholder engagement plan and risk management plan approved.
3. Development of rating shell from nuts collected in 2017
4. Progress report

Funding statement:

Levy funds – R&D projects

This project has been funded by Hort Innovation, using the <insert industry> research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

General project overview

The pistachio industry is worth up to $25 million and is dependent on the production of a quality product for export and domestic markets. The pistachio industry identified fungal diseases as a major threat to productivity and market expansion. In 2017, dark staining of pistachio shells and nuts was observed and preliminary studies recovered *Fusarium* sp. and *Alternaria* sp. from stained samples. Infected produce is unmarketable and fungal infection can cause considerable yield loss (up to 50%). It is known that other fungi including *Aspergillus sp., Colleototrichum and Botryosphaeria* can infect pistachio. Using field and laboratory studies, this project will evaluate the occurrence of dark staining in pistachio and provide information on the casual agents associated with the problem. The project will also assess if insect damage or environmental factors influence fungal infection. The information will provide a monitoring tool for growers to use in evaluating the risk of fungal disease, and assist in the future development of management strategies.

Summary

The pistachio industry is worth up to $25 million and is dependent on the production of a quality product for export and domestic markets. Dark staining of pistachio shells and nuts can cause a significant reduction in marketable product and threatens the expansion of the pistachio industry. In 2017, some orchards reported up to 50% of shells having dark staining. The cause of dark staining is unknown, although the problem may be associated with fungal infection. The aim of the project is to determine the causal organism associated with dark staining, evaluate when pistachio are most susceptible to infection and other factors associated with the incidence of dark staining. The information will provide a monitoring tool for growers to use in evaluating the risk of fungal disease, and assist in the future development of management strategies.

The Riverland and Sunraysia regions of Australia are ideally suited for pistachio growing. A central commercial processing facility is located at Robinvale, VIC. Project outcomes are on target with field trials established at Robinvale, VIC and Kyalite, NSW to monitor the development and occurrence of dark staining. Trials have been established in orchard blocks which showed previous high levels of dark staining. During the growing season, pistachio crops have been monitored for signs of fungal disease on leaves and developing fruit. Clusters showing black spots and/or dieback was collected and fungal pathogens isolated from symptomatic material. Fruit samples collected during the season showed little evidence of dark staining on the developing embryo. In most cases, *Alternaria* sp. were detected from clusters showing characteristic disease symptoms.

Five trees at each site were allocated for collection of clusters at 5-day intervals from -15, -10 and -5 days pre-harvest, optimum harvest date, and +5, +10, +15, +20 and +25 days post-harvest (or until clusters remain on the tree). Prior to the trial collection period, fruit was collected to evaluate the viability in storage for diagnosis of fungal pathogens. Commercial harvesting of pistachio commenced 26 February 2018, with samples at Robinvale and Kyalite first harvested on the 4th and 17th March, respectively. At each timing, 250 fruit were examined for the development and presence of dark staining of the shell in relation to external and internal damage/blackening of the hull. Nuts were graded according to existing industry standards, including light staining and dark staining. Dark staining occurred when hull slip was possible – i.e. the hull no longer adhered to the shell. Culturing of hull and shell material from symptomatic and non-symptomatic nuts is being conducted from each sampling period. A number of fungal pathogens were isolated from infected nuts, including *Alternaria*, *Fusarium* and *Rhizopus* sp.. Sampling is currently ongoing and pathogenicity tests will be conducted to assess the causal agent of dark staining.

Achievements

1. Meet with PGAI to agreed project plan

At the commencement of the project, Dr Rawnsley met with Chris Joyce (Research Chairperson, PGAI) and Trevor Ranford (Executive Officer, PGAI) to align planned research activities with project objectives. Contact was also made with Stuart Pettigrew, Project Leader of PS16000-Managing insects on pistachio collaboration research findings.

2. Monitoring and Evaluation Plan, Stakeholder engagement plan and risk management plan approved.
   Research of current and past literature and discussion with industry personnel assisted to development the monitoring and evaluation plan in accordance to previous history of dark staining. The proposed methodology demonstrates a clear study path to achieving the project outcomes. Dr Rawnsley attended and presented project findings to the PGAI members at Mildura in February 2018. Discussions were held to validate and provide feedback on the directions of the project to ensure research was aligned to industry priorities.

3. Development of rating shell from nuts collected in 2017
   The commercial grading of shells and kernels is well established and conducted by seasoned industry professionals for every batch of pistachios processed at the Robinvale facility. Samples have been retained from the 2017 season and assessed to ensure commercial rating of dark staining in the project. The Robinvale processing plant was visited in March 2018 once dark staining was detected on samples at the facility.

4. Progress report
   The progress report was written and submitted by the due date.

Outputs

A presentation by Dr Rawnsley was delivered to the PGAI board members on Feb 12 2018 at Mildura, VIC. The presentation outlined the research plan and methodologies, and discussion on information that would assist project outcomes. Approximately 16 people were in attendance.

Refereed scientific publications

None to report

Outcomes

The infection of pistachio fruits and bunch dieback observed during the growing season is unlikely to contribute to dark staining. Preliminary studies have shown that dark staining of shells occurs close to/at the optimum harvest date. The establishment of field trials and sampling clusters at regular intervals is building knowledge on when dark staining occurs and the role of nut development on the tree. It appears the longer the nut remains on the tree past the optimum harvest date, the increased risk of dark staining. At the conclusion of the season, the results will provide a clearer understanding of the causal agent, and what factors, such as environmental or other, are associated with dark staining.

Intellectual property, commercialisation and confidentiality

No project IP, project outputs, commercialisation or confidentiality issues to report

Issues and risks

To date, the incidence of dark staining appears significantly less than observed in 2017.

Regardless, the collection of material at the field trials has shown dark staining is occurring, and therefore adequate material is available for diagnostics. Close monitoring of sites and consultation with growers in the industry will ensure the delivery of agreed project outputs.

As provided in the Research Agreement, technical staff to assist Dr Rawnsley were listed. Changes to personnel have since occurred and the role of Georgia Megirian (Technical Officer) in assisting with lab and field studies has been replaced by Bethany Greatz.

Other information

No additional information to report

Appendices

As the season is still underway, no appendices or preliminary results have been provided.

Site:			Date:			
	No. clean hull	**No. hull outer stained**	**No. hull internal stain**	**No. dark stain shell**	**No. light stain shell**	**No. clean shells**
Tree 1						
Tree 2						
Tree 3						
Tree 4						
Tree 5						
Comments						

Appendix ix: HIA Milestone 103

Milestone Report

Project title:

Pathogens and other factors contributing to dark staining on pistachio shells

Project code:

PS16002

Milestone number:

103

Project leader:

Dr Belinda Rawnsley

Delivery partner:

AgXtra

Report author:

Dr Belinda Rawnsley

Milestone due date:

24 September 2018

Submission date:

4 October 2018

Confidentiality:

No

Milestone description:

1. Preliminary data to PGAI
2. Annual industry report to Pistachio Growers Association and 6-month milestone report

Milestone achievement criteria:

Meeting with PGAI and provision of data generated on fungal isolations from 2017/2018 season
Annual report submitted to PGAI and milestone report including annual report submitted to Hort Innovation (Annual report as appendix)

Funding statement:

Levy funds – R&D projects

This project has been funded by Hort Innovation, using the <insert industry> research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

General project overview

The pistachio industry is worth up to $25 million and is dependent on the production of a quality product for export and domestic markets. The pistachio industry identified fungal diseases as a major threat to productivity and market expansion. In 2017, dark staining of pistachio shells and nuts was observed and preliminary studies recovered *Fusarium* sp. and *Alternaria* sp. from stained samples. Infected produce is unmarketable and fungal infection can cause considerable yield loss (up to 50%). It is known that other fungi including *Aspergillus sp., Colleototrichum and Botryosphaeria* can infect pistachio. Using field and laboratory studies, this project will evaluate the occurrence of dark staining in pistachio and provide information on the casual agents associated with the problem. The project will also assess if insect damage or environmental factors influence fungal infection. The information will provide a monitoring tool for growers to use in evaluating the risk of fungal disease, and assist in the future development of management strategies.

Summary

The pistachio industry is worth up to $25 million annually in Australia, and is dependent on the production of a quality product for export and domestic markets. Dark staining of pistachio shells and nuts can cause a significant reduction in marketable product and threatens the expansion of the pistachio industry. In 2017, some orchards reported up to 50% of shells having dark staining. The cause of dark staining is unknown, although the problem may be associated with fungal infection.

The aim of the project is to determine the causal agent associated with dark staining, evaluate when pistachio is most susceptible to dark staining and other factors associated with the incidence of the problem. The information will provide a monitoring tool for growers to use in evaluating the risk of fungal disease and assist in the future development of management strategies.

A series of experiments were conducted to determine the development and timing of dark staining. Field sampling areas were established in Robinvale, VIC and Kyalite, NSW. Orchard blocks which showed previous high levels of dark staining were used to assess fruit during the season and during the harvest period.

During the growing season, pistachio trees were monitored for signs of fungal disease on leaves and developing fruit. Clusters showing black spots and/or dieback were collected and fungal pathogens isolated from symptomatic material. Fruit samples collected during the season showed little evidence of dark staining on the developing embryo. In most cases, *Alternaria* sp. were detected from clusters showing characteristic disease symptoms. Fruit that did show dark staining often did not develop and deteriorated prior to ripeness. For this reason, it was not possible to align fruit necrosis with predictions of dark staining of the shell observed at hulling.

Five selected trees were used for collection of fruit at various times throughout the commercial harvest period. Approximately every 5 days, 250 fruit were examined for the development and presence of dark staining of the shell in relation to external and internal damage/blackening of the hull. Nuts were graded according to existing industry standards, including light staining and dark staining. Dark staining was detected when hull slip occurred i.e. the hull no longer adhered to the shell. Fungal pathogens were isolated from shells with and without dark staining nuts including *Alternaria*, *Fusarium, Penicillium* and *Rhizopus* sp.. Fungi were isolated from clean shells as well as those with dark staining, therefore there was no strong correlation that dark staining was solely caused by fungi.

Fruit with adhered hulls were infected with fungi to reproduce dark staining of shells. Dark staining occurred up to 28 days after infection. Microscopic observation of dark staining of shells was dissimilar to staining caused by fungal infection. It is proposed phenological oxidation may be associated with dark staining.

The second year of research will focus on further pathology work, environmental factors, and

biochemical interactions.

Achievements

1. Preliminary data to PGAI

   Dr Belinda Rawnsley attended the Pistachio Growers Association Spring Symposium and AGM in Robinvale, Victoria on 13 September 2018. The results of project PS 16002: Pathogens and other factors contributing to dark staining on pistachio shells were presented to 55 members and industry personnel in attendance.

2. Annual industry report to Pistachio Growers Association and 6-month milestone report

   The annual industry report outlining methodologies, results and research outcomes was submitted to the Pistachio Growers Association on 3 October 2018 (see Appendix). The 6-month milestone report was submitted on 4 October 2018.

Outputs

The outputs outlined in the Monitoring and Evaluation Plan have been achieved by the attendance at pistachio grower association meetings and provision of information on current findings.

Dr Belinda Rawnsley provided a progress research report to the PGAI on 6 June 2018 and attended the Pistachio Growers Association Spring Symposium and AGM in Robinvale, Victoria on 13 September 2018. The results of project PS 16002: Pathogens and other factors contributing to dark staining on pistachio shells were presented to 55 members and industry personnel in attendance.

Refereed scientific publications

None to report

Outcomes

A series of experiments have been conducted in the first year to determine if fungal pathogens contribute the contribute to dark staining of pistachio shells. Orchard trees were monitored throughout the growing season and fruit collected at 5-day intervals pre- and post-harvest to determine when dark staining occurred. Dark staining occurred 10-12 days prior to optimal harvest date and increased with delayed time on the tree until the second harvest shake. Uniformity of fruit maturity may be a contributing factor to the increase in dark staining incidence. Laboratory experiments showed several fungi including *Alternaria*, *Fusarium*, *Penicillium* and *Rhizopus* were isolated from pistachio hulls and hells. However, fungi were isolated from both clean and stained material which did not support that fungal pathogens were solely responsible for dark staining. In addition, preliminary pathogenicity tests showed dark staining resulting from fungal infection was dissimilar to the dark staining classified by industry quality standards. It is possible phenolics may play a primary role in dark staining.

Growers and industry personnel have kept up to date of research findings through the season via presentation to committee members, industry members and personnel. Critical discussions have been held with members to validate current findings and the direction of year two research.

Intellectual property, commercialisation and confidentiality

No project IP, project outputs, commercialisation or confidentiality issues to report

Issues and risks

The incidence of dark staining was significantly less in 2018 than recorded in 2017. Regardless, the collection of material at the field trials has shown dark staining occurs 10-12 days prior to optimal harvest. Although there was limited material in terms of the number of dark stained shells for diagnostics, adequate material was used to conduct experimental work.

With the reduced incidence of dark staining and travel time to collect samples in Victoria and New South Wales, fruit samples will also be collected in South Australia in the second year of the study.

Other information

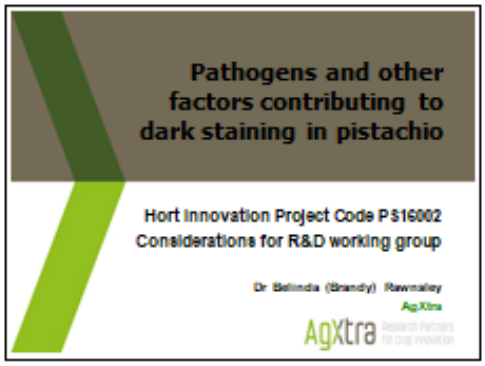
No additional information to report

Appendices

Annual Industry Report – Pathogens and Other Factors Contributing to dark staining on pistachio shells

Attached as pdf file.

Appendix x: HIA Milestone 104



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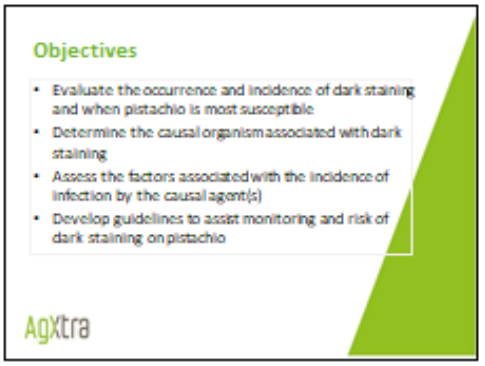

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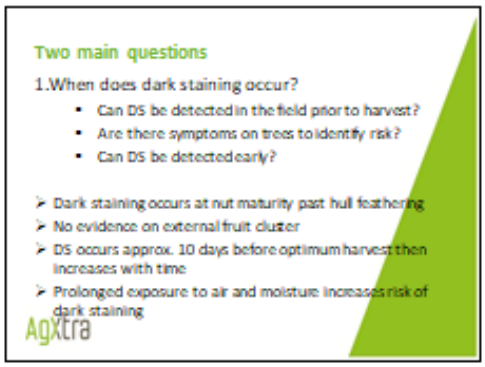

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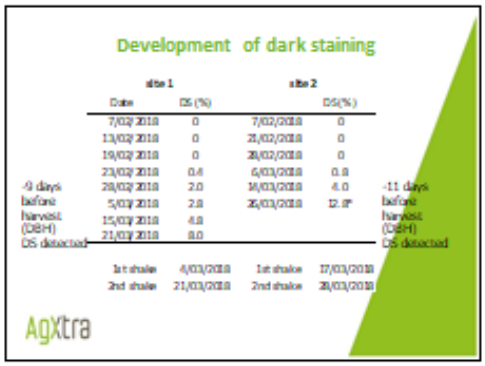

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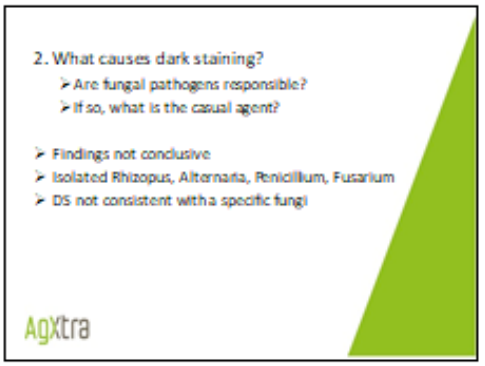

6


Severe dark stained hull > Dark stained shell
• Severe blackening of hull usually associated with fungal infection
• In many cases, nut was not viable
AgXtra

7


Clean unstained shells – natural infection Rhizopus
AgXtra

8


Dark stained shells – also had infection by Rhizopus
AgXtra

9


Natural dark staining vs dark staining from inoculation
Natural dark staining
Fungal inoculated dark staining
AgXtra

10


Collection of DS shells following de-hulling
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Specific isolation of DS shell after surface sterilisation
AgXtra

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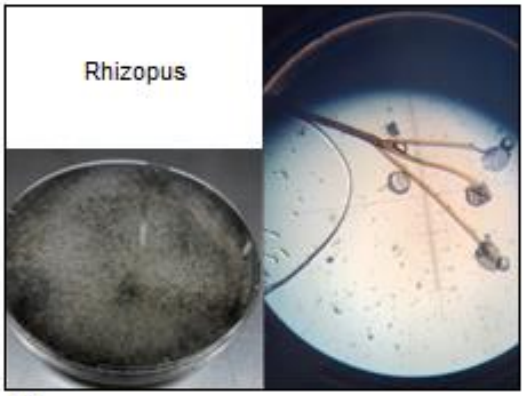

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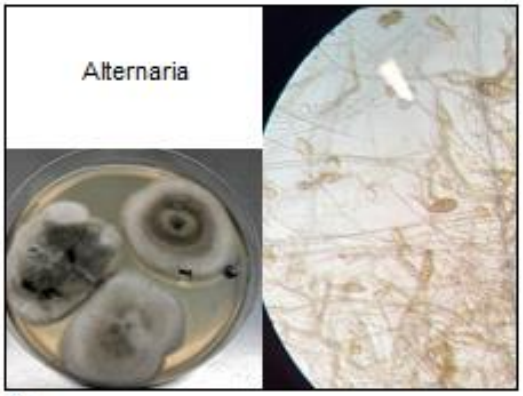

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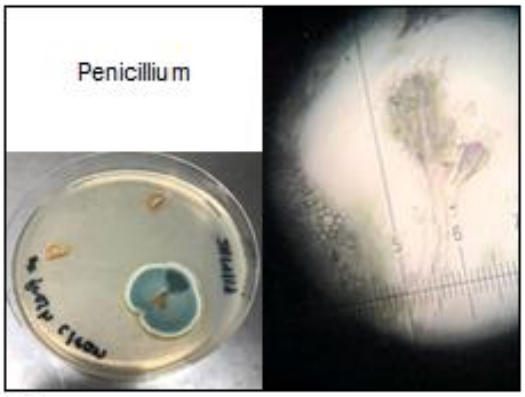

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Casual fungal pathogen – post-hulling

	Rhizopus	Penicillium	Alternaria	Fusarium
Lot 17	50	50	60	0
Lot 139	100	30	20	10
Lot 165	0	100	20	40
Lot 193	100	60	0	0
Lot 299	0	50	0	20
Lot 301	50	20	0	0
Lot 302	50	10	10	20
Lot 306	0	40	30	0
Lot 306	0	40	20	0
Lot 308	100	10	0	10
Untreated	0	20	0	0
Fungal %	45	41	16	10
Untreated %	0	20	0	0

AgXtra

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Phenolics

- Enzymatic reaction that causes browning
- Pistachios are high in phenols
- Staining can occur as result of the breakdown of phenols in pistachio
- Oxidation of phenolics upon exposure of the shell to air and moisture – polyphenol oxidation
- Presence of oxygen causes the reaction
- Polyphenolic oxidase (PPO) is being analysed by SARDI Food Innovation Analytical Laboratory (West Beach)

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What next?

- Confirm casual fungal pathogens in dried nut samples
- Confirm if polyphenolic oxidation is occurring
- With development of DS so close to harvest – what are the options?
- Plant growth regulators to improve uniformity of ripening?
- Harvest and storage conditions?
- Further funding opportunity through SA Govt Innovation Connections

AgXtra

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Appendix xiii: HIA Milestone 106

Milestone Report

Project title:

Pathogens and other factors contributing to dark staining on pistachio shells

Project code:

PS16002

Milestone number:

106

Project leader:

Dr Belinda Rawnsley

Delivery partner:

AgXtra

Report author:

Dr Belinda Rawnsley

Milestone due date:

30 September 2019

Submission date:

24 September 2019

Confidentiality:

No

Milestone description:

6-month milestone report

Milestone achievement criteria:

Milestone report submitted to Hort Innovation

Funding statement:

Levy funds – R&D projects

This project has been funded by Hort Innovation, using the Pistachio research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

General project overview

In 2018, the pistachio industry was valued at $35.4 million and is dependent on the production of a quality product for export and domestic markets. In 2018, pistachio production reached an industry record of 3,200 tonnes, up 50% from the previous year. In 2017, there was a high incidence of dark staining of shells which reduced the marketability of pistachios both domestically and overseas. The cause of dark staining however is unknown. The pistachio industry identified fungal diseases as a major threat to productivity and market expansion. Infected produce is unmarketable and fungal infection can cause considerable yield loss (up to 50%). This project was established to evaluate the occurrence of dark staining in pistachio and provide information on the casual agents related with the problem. Using field and laboratory studies, the project will assist in identifying the cause of dark staining and if fungal pathogens or environmental constraints are correlated to seasonal variability of dark staining. The information will provide a monitoring tool for growers to use in evaluating the risk of dark staining and assist in the future direction to mitigate the problem.

Summary

The major pistachio production areas are along the Murray River between Swan Hill in Victoria and Waikerie in SA with up to 3,200 tonnes produced in 2018. Dark staining of pistachio shells and nuts can cause a significant reduction in marketable product and threatens the expansion of the pistachio industry. In 2017, some orchards reported up to 50% of shells having dark staining yet in following seasons the incidence of dark staining was low. The cause of dark staining is unknown, although anecdotally the problem has been associated with fungal infection.

The aim of the project is to determine the causal agent associated with dark staining, evaluate when pistachio is most susceptible to dark staining and other factors associated with the incidence of the problem. The information will provide a monitoring tool for growers to use in evaluating the risk of fungal disease and assist in the future development of management strategies.

In the first year of research, it was determined dark staining occurs predominantly at hull slip when the hull no longer adheres to the shell. Dark staining was first detected 10 days prior to optimum harvest and the incidence increased after the first harvest shake. The severity of dark staining progressively worsens with ripe fruit left on the tree and for this reason, delayed harvesting increases the risk of dark staining.

A number of fungal pathogens including *Alternaria*, *Fusarium*, *Penicillium* and *Rhizopus* sp.. were isolated from dark stained pistachio processed nuts. In the past season, pistachios were sampled immediately post-hulling at the Australian Nut Producers processing facility in Robinvale, Vic. Samples were collected from pistachios derived from many orchard blocks throughout the harvest period. As in other seasons, the incidence of dark staining was low, with dark staining more prevalent on nuts at the end of the season. After surface sterilisation and laboratory isolation, a high proportion of dark stained

shells were found to be infected with either *Rhizopus stolonifer* or *Alternaria alternata* (28% and 56%, respectively). Although clean shells with no staining also showed fungi, the results show there is a strong correlation of fungal infection and dark staining. The incidence of dark staining may be very seasonal dependent, with environmental parameters such as high relative humidity at the time of hull slip necessary for fungal infection to occur.

To assess if other factors were contributing to dark staining, pistachios were analysed for polyphenol oxidase (PPO) activity, which is associated with browning of fruit. Nuts were categorized into varying degrees of dark staining, yet the enzyme PPO was detected in shells regardless of the level of staining and with no staining at all.

Achievements

In the first year of research, the project improved knowledge of dark staining of pistachio shells and developmental time. Fruit was collected from trees at various times in the orchard in accordance to predicted maturity and harvest times. Collections every 5-7 days from ripening to 2 weeks post-harvest showed dark staining occurred predominantly at hull slip when the hull no longer adhered to the shell. Dark staining was first detected approximately 10 days prior to optimum harvest and the incidence increased after the first harvest shake. This has assisted growers in the pistachio industry to identify the key risk time for dark staining to occur. Early detection of dark staining can be used as a guide for harvesting priorisation. The project results showed the severity of dark staining progressively worsens with ripe fruit left on the tree and for this reason, delayed harvesting increases the risk of dark staining.

In order to determine the cause of dark staining, shells with dark staining were assessed and fungi directly isolated in culture. In plant pathology terms, Koch's postulates must be followed to show that the isolated fungi must be derived from symptomatic tissue (dark stained shell) and be associated with the symptom by re-infecting new fruit. A number of fungal pathogens including *Alternaria*, *Fusarium*, *Penicillium* and *Rhizopus* sp.. were isolated from dark stained pistachio processed nuts. Because fungi were also detected in unstained marketable fruit, further studies were undertaken in the second year of the project to sample pistachios immediately post-hulling at the Australian Nut Producers processing facility in Robinvale, Vic. Samples were collected from pistachios derived from many orchard blocks throughout the harvest period to show spatial differences, if any. As in other seasons, the incidence of dark staining was low, with dark staining more prevalent on nuts at the end of the harvest season. The data showed dark staining was highly corelated to maturity and ripening time. After surface sterilisation and isolation, a high proportion of dark stained shells were found to be infected with either *Rhizopus stolonifer* or *Alternaria alternata* (28% and 56%, respectively). The results, although not conclusive, indicate fungal pathogens may be associated with dark staining of pistachio but infection is highly dependent on conducive environmental conditions. Weather data from past seasons has been compiled to assess if there is a correlation with temperature, rainfall and relative humidity. The 2017 season was not considered wet, however relative humidity at hull slip may have been an influencing factor in the development of dark staining.

To gain a greater understanding of dark staining and associated possible fungal pathogens, histological studies will ascertain if fungi invade the pistachio shell or cause a break down of cell tissue. In the next season, pistachio clusters will be inoculated with fungi isolated from dark stained shells under conditions conducive for disease development. This line of study will support whether fungi cause dark staining and ultimately assist future management strategies.

The project activities have been undertaken systematically, with the experimental plan considering new findings. With the onset of dark staining at hull slip, it is a time when oxygen and moisture are exposed to the nut shell, which are associated with common browning of fruit. To assess if other environmental factors contributed to dark staining, pistachios were analysed for polyphenol oxidase (PPO) activity. Nuts were categorized into varying degrees of dark staining and compare to unstained shells. The enzyme PPO was detected in shells regardless of the level of staining and with no staining at all, suggesting PPO activity occurred with the breakdown of the cell tissue. There was no evidence to suggest dark stained shells had a higher level of PPOs.

Outputs

The project activities align with the monitoring and evaluation plan and outputs have been delivered as

scheduled. Data generated throughout the duration of the project has been directly communicated to the Pistachio Growers Association. Dr Rawnsley has attended the Pistachio Research Committee meetings held on 2 May, 2019, Adelaide, SA; 7 June 2019, Mildura, Vic.; and 10 September 2019, Mildura, Vic.

A key deliverable was achieved in this milestone period to disseminate project findings through oral presentation to the growers and industry representatives. A research update was presented at the Pistachio Growers Spring Symposium on 11 September 2019 at Mildura, Vic. The meeting was attended 35 growers and industry personnel.

An annual report has been submitted at the time of this milestone submission to the Pistachio Growers Association.

An article will be published in the December issue of the leading nut producers industry publication 'The Nutgrower'.

Refereed scientific publications

None to report

Outcomes

Significant progress has been made with field collections and laboratory experiments towards achieving the designated project outcomes and improving grower knowledge of dark staining. Information has been relayed to industry members on the development of dark staining and the key times when it occurs. Comprehensive field collections showed dark staining occurred 10-12 days prior to optimal harvest date and increased with delayed time on the tree until the second harvest shake. This information assisted the revision of project activities to generate comprehensive data at harvest when the incidence of dark staining is greater.

The project findings have also broadened the scope of the project to consider environmental and biochemical reactions such as PPO activity that may be correlated with pathological factors associated with dark staining. Laboratory experiments showed several fungi including *Alternaria*, *Fusarium*, *Penicillium* and *Rhizopus* were isolated from pistachio hulls and shells, and histological studies may support fungal infection as a direct consequence of dark staining.

Intellectual property, commercialisation and confidentiality

No project IP, project outputs, commercialisation or confidentiality issues to report

Issues and risks

With the incidence of dark staining significantly less during the project period than observed in 2017, there have been fewer pistachio samples to analyse than anticipated. The planned project methodology has been modified from the initial project plan to ensure adequate dark stained material is available for experimental work. Inoculation studies will further improve our understanding of the cause of dark staining.

Other information

No additional information to report

Appendices

Appendix i

Pistachio PPT PS16002 Spring Symposium 11 Sept 2019

Appendix xv: PGAI 6-month milestone progress report 2020 107

Milestone Report

Project title:

Pathogens and other factors contributing to dark staining on pistachio shells

Project code:

PS16002

Milestone number:

107

Project leader:

Richard Porter

Delivery partner:

AgXtra

Report author:

Barbara Hall

Milestone due date:

30/03/2020

Submission date:

09/04/2020

Confidentiality:

Is this report confidential?

No

Milestone description:

1. Field and inoculation studies.
2. 6 month milestone report

Milestone achievement criteria:

1. Milestone report submitted to HIA

Funding statement:

Levy funds – R&D projects

This project has been funded by Hort Innovation, using the pistachio research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

General project overview

The pistachio industry is worth up to $25 million annually and is dependent on the production of a quality product for export and domestic markets. The pistachio industry identified fungal diseases as a major threat to productivity and market expansion. In 2017, dark staining of pistachio shells and nuts was observed and preliminary studies recovered *Fusarium* sp. and *Alternaria* sp. from stained samples. Infected produce is unmarketable and fungal infection can cause considerable yield loss (up to 50%). It is known that other fungi including *Aspergillus sp., Colletotrichum and Botryosphaeria* can infect pistachio. Using field and laboratory studies, this project will evaluate the occurrence of dark staining in pistachio and provide information on the casual agents associated with the problem. The project will also assess if insect damage or environmental factors influence fungal infection. The information will provide a monitoring tool for growers to use in evaluating the risk of fungal disease, and assist in the future development of management strategies.

Summary

The pistachio industry is worth up to $25 million annually and is dependent on the production of a quality product for export and domestic markets. Dark staining of pistachio shells and nuts can cause a significant reduction in marketable product and threatens the expansion of the pistachio industry. In 2017, some orchards reported up to 50% of shells having dark staining. The cause of dark staining is unknown, although the problem may be associated with fungal infection. The aim of the project is to determine the causal organism associated with dark staining, evaluate when pistachio are most susceptible to infection and other factors associated with the incidence of dark staining. The information will provide a monitoring tool for growers to use in evaluating the risk of fungal disease, and assist in the future development of management strategies.

Achievements

1. Field and inoculation studies
    - 13 November 2019: Article submitted to NutGrower magazine for publication
    - 25-28 November 2019: Dr Belinda Rawnsley presented to scientists at the Australian Plant Pathology Society Biennial Conference on the project, with an oral presentation and a poster.
    - Jan-21-2020: Prior to the expected pistachio harvest, Bethany Greatz and Richard Porter met with grower Ian Mau of Mallee Nuts to assess trees and discuss trial set up and location where a weather monitoring station was also installed to record weather data over the trial period.
    - Feb-04-2020: Report presentation to PGAI R&D committee.
    - Feb-06-2020: *Rhizopus*, *Penicillium* and *Alternaria* inoculum plates made up by Bethany Greatz from cultures previously recovered from pistachio nuts.
    - Feb-17-2020: Pistachio clusters tagged for later inoculation closer to expected harvest. 25 replicate clusters with 25-40 fruit each were tagged for each of the 10 treatments as per agreed trial plan. No symptoms of *Botryosphaeria* or *Alternaria* were evident on leaves or bunches.
    - Feb-27-2020: Tagged clusters were inoculated with spore suspensions of either *Alternaria*, *Rhizopus*, or *Penicillium* 10-15 days prior to expected first harvest (just prior to hull split). Clusters were sprayed until wetted with spore suspension, placed in clear plastic bags and left for 10 hours to induce and sustain a humid microenvironment, providing conditions conducive for fungal infection.
    - Feb-28-2020: Bags were removed from tagged clusters at sunrise to avoid exposure to sun.
    - Mar-16-2020: 1st harvest. Clusters were covered with mesh bags to collect fruit when harvested. Trees were machine harvested by Ian Mau and bags containing sample fruit were removed and collected for assessment in the laboratory.
    - From Mar-18-2020: 25 subsamples of nuts were taken from each replicate sample and hulls were assessed and categorized by deterioration. Samples were then hulled and subcategorized by shell characteristics; clean, grey staining, light staining and dark staining.
    - Mar-27-2020: Just prior to advised 2nd harvest remaining tagged clustered were collected from trees and brought back to the laboratory for assessment.
    - Mar-29-2020 to Mar-30-2020: remaining ripe subsamples were assessed from each sample and classified by the deterioration of the hull and presence of light or dark staining.

- Mar-31 to Apr-01-2020: subsamples from 1st and 2nd harvest with symptoms of light or dark staining were placed into humid trays to create conditions conducive for fungal growth.
- Apr-06-2020 to present: Assessment of subsamples for pathogen completed by Barbara Hall.

Outputs

A presentation by Dr Belinda Rawnsley was delivered to the PGAI board members on Sep 10 2019 at Mildura, VIC. The presentation outlined the research plan and methodologies, and discussion on information that would assist project outcomes.

In November 2019 Dr Belinda Rawnsley presented orally and with a poster to the Australian Plant Pathology Society Biennial conference.

On February 4th a report was tabled to PGAI R&D committee, with Barbara Hall, Richard Porter and Bethany Greatz supporting the report via tele conference.

An article on the project was submitted on 13th November to the NutGrower magazine for publication.

Refereed scientific publications

None to report

Outcomes

The infection of pistachio fruits and bunch dieback observed during the growing season is unlikely to contribute to dark staining. Preliminary studies have shown that dark staining of shells occurs close to/at the optimum harvest date. The establishment of field trials and sampling clusters at regular intervals is building knowledge on when dark staining occurs and the role of nut development on the tree. It appears the longer the nut remains on the tree past the optimum harvest date, the increased risk of dark staining. At the conclusion of the season, the results will provide a clearer understanding of the potential factors, such as fungal infection, environmental or other, are associated with dark staining.

Intellectual property, commercialisation and confidentiality

No project IP, project outputs, commercialisation or confidentiality issues to report

Issues and risks

To date, the incidence of dark staining appears significantly less than observed in 2017. Regardless, the collection of material at the field trials has shown dark staining is occurring, and therefore adequate material is available for diagnostics. Close monitoring of sites and consultation with growers in the industry will ensure the delivery of agreed project outputs.

As provided in the Research Project Variation Request, there has been changes to project personnel following the resignation of Dr Belinda Rawnsley in 2019. Richard Porter has assumed the role of Project Leader, Bethany Greatz remains as the lead Technical Officer, with significant support from plant pathologist Barbara Hall for both roles.

Other information

No additional information to report

Appendix xiv: HIA Milestone 108

Pathogens and other factors contributing to dark staining in pistachio

Hort Innovation Project Code PS16002
Research Update

Barbara Hall/Bethany Greatz
29 September 2020

Hort Innovation

AgXtra Research Partners for Crop Innovation

1

Background

- The research project began in 2017 headed by Dr Belinda (Brandy) Rawnsley with the aim to determine the causal agent associated with dark staining of pistachios and other factors associated with the incidence, eg. environmental, soil and plant conditions
- A series of experiments were conducted to determine development and timing of DS.

AgXtra

2

Previous findings

- Dark staining occurs 10-12 days prior to optimum harvest
- Incidence increases with time on tree and exposure to air and moisture
- No symptoms on external fruit cluster
- *Alternaria, Rhizopus* and *Penicillium* were predominant fungi found on both clean and stained nuts
- Inoculation did not produce same symptoms
- No correlation found between polyphenolic oxidase (PPO) and DS.

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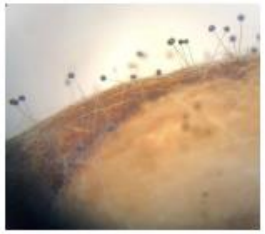
Current year objectives



Determine whether fungi are associated with dark staining through inoculation of fruit on trees.

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Site selection

Separate block at Mallee Nuts orchard located at Peebinga, South Australia used as trial site.

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5

Treatments

No.	Treatment	Bunch type
1	Untreated control - no water not bagged	Open cluster
2	Untreated control - no water not bagged	Closed tight cluster
3	Untreated control – water only	Open cluster
4	Untreated control – water only	Closed tight cluster
5	*Alternaria*	Open cluster
6	*Alternaria*	Closed tight cluster
7	*Rhizopus*	Open cluster
8	*Rhizopus*	Closed tight cluster
9	*Penicillium*	Open cluster
10	*Penicillium*	Closed tight cluster

AgXtra

6

Inoculation

- 25 treatment replicates were spread across 33 trees
- Tagged clusters on trees were inoculated with spore suspensions 27th February.

AgXtra

7

2020 Harvest assessments

- 25 random subsamples from each treatment replicate were graded on hull condition
- Nuts hulled and graded for staining

- Remaining clusters were hand-harvested just prior to second harvest and ripe nuts graded.

AgXtra

8

2020 Harvest assessments

Grey discolouration also assessed.

AgXtra

9

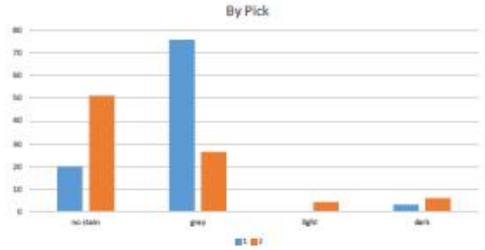
2020 Harvest results

		% nuts with staining							
		16/03/2020 (1st harvest)				27/03/2020 (2nd harvest)			
Treatment	Cluster type	No stain	Grey	Light stain	Dark stain	No stain	Grey	Light stain	Dark stain
Untreated control not bagged	Open	19.4	75.2	0.16	5.1	47	31	5	5
Untreated control not bagged	Closed	24.5	70.5	0.16	5.0	63	24	6	2
Untreated control bagged	Open	21.0	76.1	0.48	2.4	75	13	3	5
Untreated control bagged	Closed	20.1	77.2	0.32	2.4	57	18	5	12
Alternaria	Open	15.3	78.9	0	1.8	33	53	0	6
Alternaria	Closed	17.9	80.1	0.16	1.8	62	18	3	4
Rhizopus	Open	20.1	77.0	0.16	3.1	59	23	3	3
Rhizopus	Closed	20.3	76.5	0.16	3.4	31	38	5	10
Penicillium	Open	21.5	71.6	0	2.9	50	28	3	7
Penicillium	Closed	20.1	76.5	0.32	3.1	36	18	11	7

AgXtra

10

2020 Harvest results



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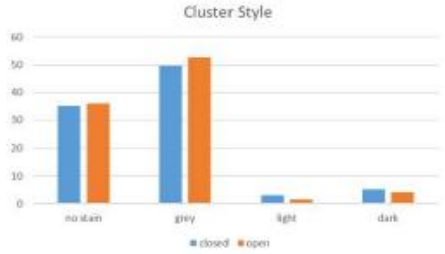
2020 Harvest results



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Incubation of stained nuts

- Samples of stained nuts were taken from treatments for incubation and mycelial growth was assessed.

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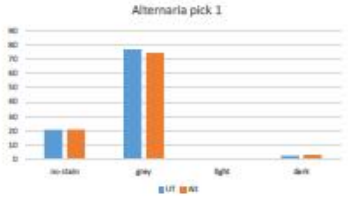
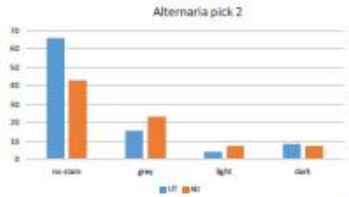
Alternaria





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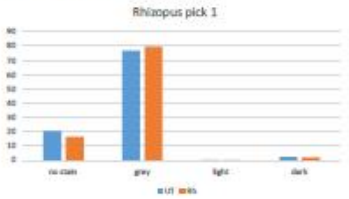
Rhizopus



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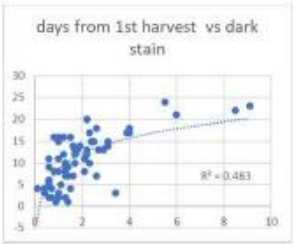
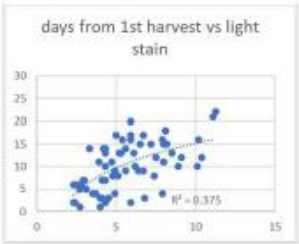
2020 Harvest

Weak correlation between days from harvest and staining.





Harvest data supplied by MOP.

AgXtra

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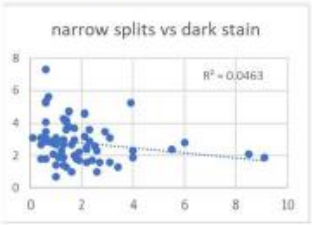
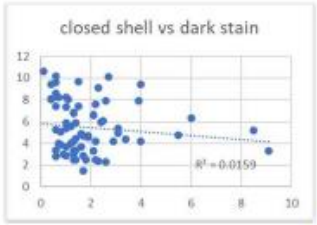
2020 Harvest

No or negative correlation between external appearance and dark stain.





AgXtra

17

Cause of DS inconclusive

- No clear correlation between external appearance and DS
- No correlation between any inoculated pathogens and incidence of DS
- Fungi most likely saprophytic
- Link with humidity would also impact saprophytic growth.

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18

Recommendations

- Environmental conditions – climate data mining
- Pattern of stain in tree and orchard
- Crop physiology, canopy management and harvest times
- Tannins – link to environmental conditions?

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19

Pathogens and other factors contributing to dark staining in pistachio

Hort Innovation Project Code PS16002

Research Update

Barbara Hall/Bethany Greatz

30 September 2020

Hort Innovation

AgXtra Research Partners for Crop Innovation

1

Background

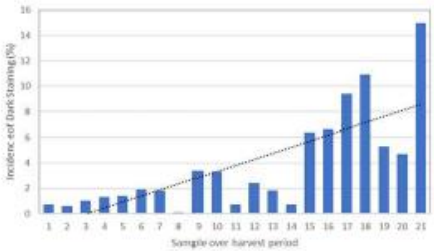
- The research project began in 2017 headed by Dr Belinda (Brandy) Rawnsley with the aim to determine the causal agent associated with dark staining of pistachios and other factors associated with the incidence, eg. environmental, soil and plant conditions
- A series of experiments were conducted to determine development and timing of DS

AgXtra

2

Timing of dark stain development

Dark stain started ~10 days prior to optimum harvest and increased with time.



Incidence of dark staining across the 2019 harvest season (1/3/2019-30/3/2019)

AgXtra

3

External symptoms

- No correlation between external symptoms on tree and dark stain.

Treatment	no stain	light	dark
	Percent of total nuts		
Intact hull	55.2	0.257	0.616
Minor scurfing	25.5	0.716	1.621
Hull damage	6.3	1.346	2.356

- Blackened nuts more likely to have dark stain but clean hulls also affected.

AgXtra

4

Fungi associated with staining

Alternaria, Rhizopus, Penicillium and *Fusarium* recovered from both clean and stained nuts.

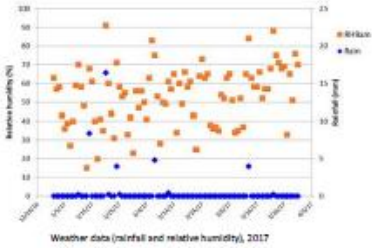
Shell	Rhizopus	Alternaria
Clean shells	24%	38%
Dark stained	28%	56%

AgXtra

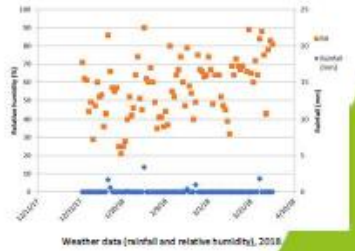
5

Does weather play a role with fungal infection?

No obvious weather event occurred that would influence fungal infection.



Weather data (rainfall and relative humidity), 2017



Weather data (rainfall and relative humidity), 2018

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Are fungal infections the cause of dark stain?

Field trial 2019-2020 season at Peebinga, SA

No.	Treatment	Bunch type
1	Untreated control - no water not bagged	Open cluster
2	Untreated control - no water not bagged	Closed tight cluster
3	Untreated control – water only	Open cluster
4	Untreated control – water only	Closed tight cluster
5	*Alternaria*	Open cluster
6	*Alternaria*	Closed tight cluster
7	*Rhizopus*	Open cluster
8	*Rhizopus*	Closed tight cluster
9	*Penicillium*	Open cluster
10	*Penicillium*	Closed tight cluster

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Inoculation

- 25 treatment replicates were spread across 33 trees
- Tagged clusters on trees were inoculated with spore suspensions 27th February

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2020 Harvest

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2020 Harvest assessments

- 25 random subsamples from each treatment replicate were graded on hull condition
- Nuts hulled and graded for staining

- Remaining clusters were hand-harvested just prior to second harvest and ripe nuts graded

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2020 Harvest results

		% nuts with staining							
		16/03/2020 (1st harvest)				27/03/2020 (2nd harvest)			
Treatment	Cluster type	No stain	Grey	Light stain	Dark stain	No stain	Grey	Light stain	Dark stain
Untreated control not bagged	Open	19.4	75.2	0.16	5.1	47	31	5	5
Untreated control not bagged	Closed	24.5	70.5	0.16	5.0	63	24	6	2
Untreated control bagged	Open	21.0	76.1	0.48	2.4	75	13	3	5
Untreated control bagged	Closed	20.1	77.2	0.32	2.4	57	18	5	12
Alternaria	Open	15.3	78.9	0	1.8	33	53	0	6
Alternaria	Closed	17.8	80.1	0.16	1.8	62	18	3	4
Rhizopus	Open	20.1	77.0	0.16	3.1	59	23	3	3
Rhizopus	Closed	20.3	76.5	0.16	3.4	31	38	5	10
Penicillium	Open	21.5	71.6	0	2.9	50	28	3	7
Penicillium	Closed	20.1	76.5	0.32	3.1	36	18	11	7

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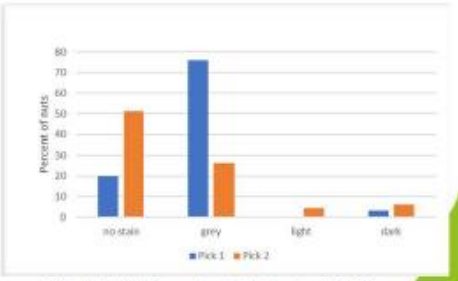
2020 Harvest results

Later pick showed more stain.



Levels of staining in first and second harvests, all treatments combined, 2020

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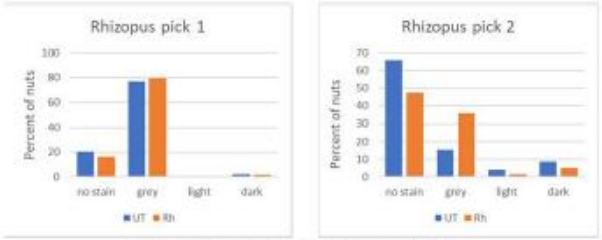
2020 Harvest results

Inoculation of fungi did not increase dark or light staining but did affect grey discolouration.



Levels of staining in first and second pick in clusters inoculated with *Rhizopus*

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Incubation of stained nuts

- Samples of stained nuts were taken from treatments for incubation and mycelial growth was assessed

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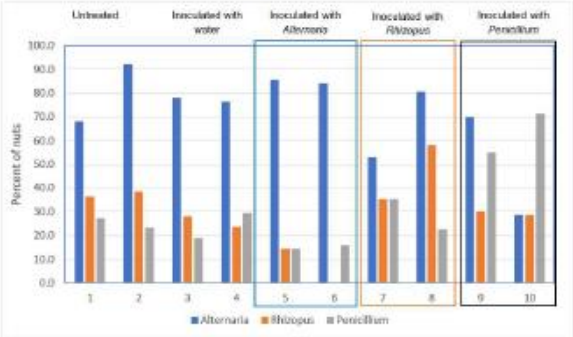
Incubation of stained nuts



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Conclusions

- Dark stain occurs close to harvest and increases with time
- External hull appearance cannot be used as a definitive indication of presence of dark stain
- Fungi detected during staining are unlikely to be the cause of the staining but are saprophytes on the damaged tissue

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Dark staining on pistachio shells – physiological or pathogenic?

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Dark staining of pistachio shells and nuts can cause significant reduction in marketable produce. With most pistachios sold in shell, the shell quality is of utmost importance. Whilst a level of dark staining is detected every year, in 2017 some orchards reported up to 50% of shells affected by dark stain. As the area of pistachio increases and the crop size increases, even small levels of dark stain can have a significant economic impact. In 2018, the Pistachio Growers Association Incorporated (PGAI) appointed AgXtra to explore the possible causal agent behind dark staining.

Between 2017 and 2020 a series of investigations were undertaken to ascertain the possible contributing factors behind dark staining including; assessment of environmental and plant conditions, polyphenol oxidase (PPO) testing, susceptible timing for infection and appearance of dark stain; histological studies and possible pathogenic causes.

Results indicated the key time for development of dark stain occurred 10-12 days prior to optimal harvest and increased on nuts with prolonged time on the tree. Several fungi, the main ones being *Alternaria, Penicillium* and *Rhizopus,* were commonly isolated from shells and hulls of collected nuts, both with and without staining. However, findings were inconclusive as to whether these fungi were responsible for dark staining.

Over the 2019-20 season, field inoculation studies of these three fungi were completed at a commercial property located at Peebinga, cv. Siora to further determine the role of fungal pathogens in dark staining. At 10-15 days prior to expected commercial harvest, clusters were inoculated by spraying with a spore suspension of the fungi and bagging overnight to induce high humidity and encourage infection (Figure 1). Two controls were included, untreated clusters and bagged clusters. As the inoculated cluster were bagged overnight, a control was included where the clusters were also bagged but only sprayed with water, to ensure this action was not affecting the outcome. Inoculated and control clusters were collected at first and second shake on 16th and 27th March respectively and returned to the laboratory, where nuts were assessed first for hull damage, then hand hulled and assessed for presence of dark and light staining (Fig 2a, b). In addition the presence of a grey discoloration was also recorded (Fig 3).

No clear correlation was found between any of the three fungi and incidence of dark staining (Table 1). The levels of grey discoloration were high, but also not affected by the fungi. Of the treatments, the highest level of dark staining was found on clusters that had not been inoculated with fungi or bagged overnight. This indicated the fungi applied at that stage had no impact on the development of dark staining. These fungi are also saprophytic or opportunistic fungi, and are most likely feeding on previously damaged tissue rather than being the causal agent of dark staining. It was also evident that damaged hulls would be more likely to contain stained shells, though dark staining was also present on shells from hulls with no external damage. Therefore, hull condition was not necessarily an indication of dark stained nuts.

Where to from here? As the cause of dark staining is still unknown but is shown not to be pathogenic, further investigations into environmental and physiological aspects such as climate data, crop management, tannins and possible patterns related to staining in orchards may prove fruitful.

Figure 1. Bagged cluster

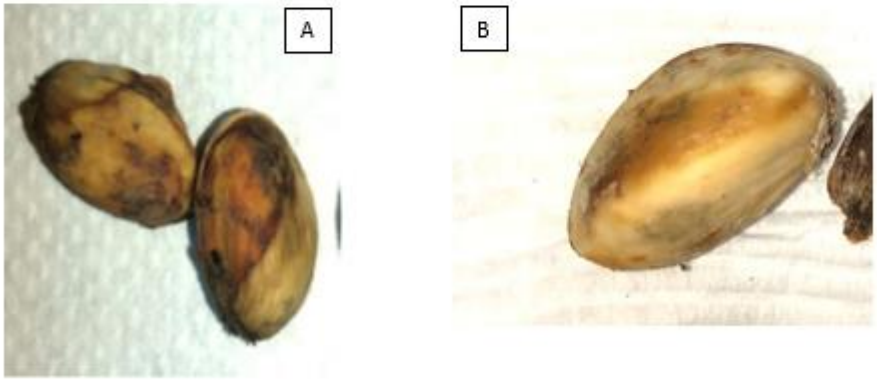

Figure 2. Typical dark staining (a) and light staining (b) as seen on post-harvest sample nuts

Figure 3. Grey discoloration noted on many nuts

Table 1. Assessment of dark staining on shells.

Treatment	no stain	grey	light	dark
	Percent of total nuts			
UT not bagged	21.96	72.82	0.16	5.06
UT bagged	20.55	76.65	0.4	2.41
Rhizopus	20.81	74.06	0.16	2.97
Penicillium	16.59	79.52	0.08	1.81
Alternaria	20.2	76.75	0.16	3.21
Tukey's HSD P=.05	9.821	10.554	0.499	3.159

Hort Innovation | PISTACHIO FUND

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